Risk Assessment of Linear Alkylbenzene Sulfonate (LAS) Based on Molecular Biomarkers

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LIST OF ABBREVIATIONS

14-3-3T: 14-3-3 protein theta

2-DE: Second dimension electrophoresis

AS: Anionic surfactants

BOD: Biochemical oxygen demand

CALR: Calreticulin

CBB: Coomassie Brilliant Blue

cDNA: Complementary DNA

COD: Chemical oxygen demand

DCF: 2',7'-dichlorofluorescein

DMEM: Dulbecco's modified Eagle's medium

DNA: Deoxyribonucleic acid

DPP3: Dipeptidyl peptidase 3

DTT: Dithiothreitol

EF2: Elongation factor 2

EPA: US Environmental Protection Agency

FAO: Food and Agriculture Organization of the United Nations

FBS: Fetal bovine serum

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GC/MS: Gas chromatography/mass spectrometry

HSP70: 70 kilodalton heat shock proteins

HSP7C: Heat shock cognate 71 kDa protein

HSP90: Heat shock protein HSP 90

IEF: Isoelectric focusing

IPCS: International Programme on Chemical Safety

LAS: Linear alkylbenzene sulfonate

LC_{50}: Lethal concentration that reduce half of the sample population of a specific test

MBAS: Methylene blue active substances

MBR: Membrane bioreactor

mRNA: Messenger RNA

MTT: 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SD: Standard deviation

SDS: Sodium dodecyl sulfate

TER: Transepithelial electrical resistance

THIO: Thioredoxin

TRPV1: Vanilloid receptor subtype 1

TSS: Total suspended solids

WHO: World Health Organization

WW: Wastewater

WWTP: Wastewater treatment plant

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CHAPTER 1

General introduction

1. Water problems in arid an semi-arid regions

Water resources scarcity has become a worldwide attention-grabbing problem having impact on political and socio-economical levels. Arid and semi-arid regions are the most concerned with that threat as shown in world map for freshwater availability, released by the FAO (Food and Agriculture Organization of the United Nations) in 2008.

Another serious issue that aggravates water problems, but unfortunately still not catching the required attention, is water pollution and quality degradation. The industrial development contributed enormously to the water contamination via the release of a huge amount of wastewaters highly loaded with mixtures of diversified pollutants, increasing thereby the associated hazards and water quality degradation (Ajibola et al., 2014; Zhang et al., 2014). This increasing water pollution boosted the development of wastewater treatment processes in order to reduce the impact on the water resources contamination and to save the remaining resources via considering new solutions such as water reuse, especially in agriculture (Khan et al., 2014; Takabe et al., 2014). However, to decide whether the water quality can cause a potential harm to environment, methods for the evaluation and assessment of associated risk with water pollution need to be developed and improved as well (Barrington et al., 2014; Peng et al., 2013). Thus, the real challenge is to provide reliable and efficient risk assessment processes that help in taking the good and appropriate decisions for risk management to stop the deterioration of water quality and save the limited resources.

2. Anionic surfactants water contamination

Surfactants are organic amphiphilic chemicals that reduce surface tension in water and other liquids. There are usually classified by their ionic behaviour in solutions: anionic, cationic, non-

ionic or amphoteric. Anionic surfactants contain anionic functional groups at their head (hydrophilic end). These surfactants are mainly used in different detergency cleaning and cosmetic products such as soaps, laundry detergents, dishwashing liquids and shampoos. They are also used in some other processes such as textile processing, mining flocculates, petroleum recovery.

Because of the extensive application of these compounds, a considerable amount of anionic surfactants are released into the environment causing serious pollution of different water compartments. Anionic surfactants can enter surface waters mainly through discharge from industries and household usage. They can cause even at low concentrations serious environmental pollution and toxicological effects on living organisms (Pavlić et al., 2005; Sobrino-Figueroa, 2013).

Even though huge efforts were dedicated to the development of wastewater treatment processes, the efficiency of these purification processes regarding anionic surfactants in the effluents, still far from perfect, resulting in the contamination of water environments by anionic detergents, raising thereby the importance of this kind of pollution and its risk assessment.

This emerging form of water pollution is becoming more relevant in the developing counties of the arid and semiarid regions, such as Tunisia, where more than 100000 m^3/year of surfactant containing wastewaters are released (Dhouib et al., 2005).

3. Linear Alkylbenzene Sulfonates

Linear alkylbenzene sulphonate (LAS) is a synthetic anionic surfactant. LAS is a mixture of closely related isomers and homologues, each containing an aromatic ring sulphonated at the para position and attached to a linear alkyl chain (**Fig. 1. 1.**). It is the primary cleaning agent used

in many laundry detergents and cleaners at concentrations up to 25 percent in consumer products, and up to 30 percent in commercial products (SIDS INITIAL ASSESSMENT REPORT, 2005).

Regarding its intensive production and use, 390,000 metric tons of LAS were consumed in North America in 2000 (Colin A. Houston, 2002), while its production in Japan and Europe in the same year was approximately 85,000 and 400,000 metric tons respectively (HERA, 2009). Finally, the most recent data available on global production was 2.6 million metric tons (EU Risk Assessment Report for LAB, 1997). Based on the results of the survey of SIDS Coalition (LAS SIDS Coalition Survey 2002), about 78-97% of the LAS consumption worldwide is in liquid and powder consumer and industrial laundry and fine fabric detergents.

Following use, the predominant disposal route for these products is via the wastewater. Products containing LAS disposed of down-the-drain are transported to wastewater treatment plants or discharged to the environment. Consequently, Environmental releases from down-the-drain discharges could lead to potential ecological exposures in surface waters and possibly in agricultural soils. LAS not biodegraded in wastewater treatment will be discharged in effluent or in the sludge produced by wastewater treatment process. The concentration of LAS in raw sewage range from 1-21 mg/l however in the treated effluent it decreases to a range of 0.01-5.91 mg/ml, depending on the treatment process used (Mungray and Kumar, 2009).

Furthermore, The presence of LAS in many commonly used household detergents gives rise to a variety of possible consumer contact scenarios including direct and indirect skin contact, inhalation, and oral ingestion derived either from residues deposited on dishes, from accidental product ingestion, or indirectly from drinking water.

The toxicological data show that LAS is toxic compound to different terrestrial and aquatic living organisms but not genotoxic/tumorigenic *in vitro* or *in vivo* (Debelius et al., 2008; Krogh et al., 2007; Marin et al., 1991).

Taken together, the risk assessment of this compound seems to be of a great importance, and accordingly the focus of the present work was on LAS.

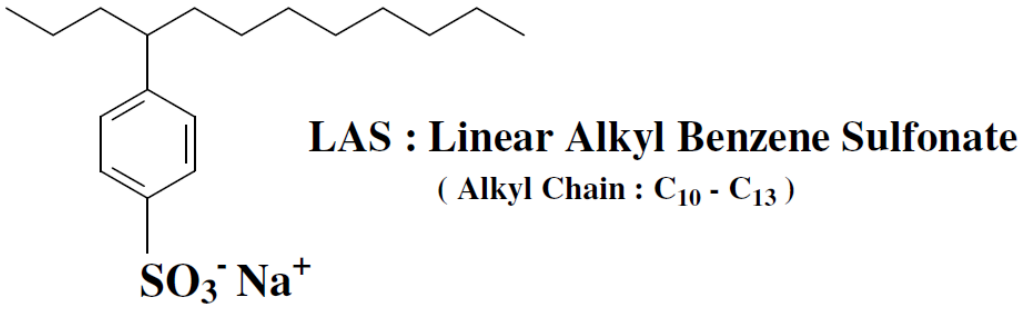


Fig. 1. 1. Typical structural formula of Linear Alkylbenzene Sulfonate (LAS)

4. Challenges for environmental risk assessment

Environmental risk assessment is the most important step toward an adequate and efficient risk management, capable of minimizing environmental impact, impacts to humans, and maximizing sustainability. Therefore, the process of risk assessment is continually developing and evolving in an effort to address changing needs. Consequently, current process is encountering many challenges to overcome its limits. In fact, the current risk assessment process is interested in the active substances taken separately, putting the emphasis on the concentration of the pollutant alone, which does not reflect necessarily its real effect on the endpoint level, especially when considering interactions and cumulative effects in environmental pollutants mixture.

Thus, the development and improvement of risk assessment procedures for combined and mixed exposures is an issue of many authorities world-wide (WHO, USA and the European Union) (Commission of the European Union, 2009; U.S. Environmental Protection Agency, 2003). Recently, these authorities emphasized the need to consider combined and mixed exposures of chemicals in future risk assessments, and to further develop legislation, methodology, and to support research within this area (Silins and Högberg, 2011).

To overcome these limits, emphasis should be directed to the toxicological effect, with an increased understanding and knowledge about the individual agents, uptake, metabolism, excretion and mechanisms/modes of action. Even though there has been recent progress within this area of research, the development of risk assessment approaches is still restrained by lack of data (National Academies Standing Committee on the Use of Emerging Science for Environmental Health Decisions, 2012).

One way to tackle these challenges is to deal holistically with the adverse effects of exposures by including biomarkers in the risk assessment process (Galloway, 2006). Suites of biomarkers encompassing molecular change, cellular pathology and physiological impairment can be developed and adapted for human and ecological scenarios. This can help risk assessment to develop the efficiency, reliability and predictive power to adapt to the unforeseen environmental threats.

5. Biomarkers

Over the last decades, the field of biomarkers has greatly expanded as early and specific endpoints for monitoring cellular responses to various disease states and exposures to xenobiotics and chemical agents. They were widely applied as successful predictors of health problems for some clinical diseases, but their application to chemical exposure risk assessments has been more limited (Fowler, 2012).

Currently, “omics” biomarkers (i.e., genomic, proteomic, and metabolomic) are the major classes of biomarkers under development (Fowler, 2012). These biomarkers reflect a variety of cellular responses to xenobiotics or toxicants exposures and interestingly provide linkages to, and insight into, mechanisms of cell injury/cell death or carcinogenic transformation, as well as the xenobiotic or toxicant mode of action (Chiu et al., 2013).

One way to tackle the recommendations of WHO, IPCS and US EPA, in order to develop a framework for the integrated assessment of human and environmental risk assessments improving the quality, efficiency and predictive capability of existing procedures (Munns et al., 2003), is to deal holistically with the adverse health consequences of exposures by including biomarkers in the risk assessment process (Galloway, 2006).

6. Importance of *in vitro* systems in toxicology: Caco-2 cell model

During the last few decades, *in vitro* studies of cell and tissue cultures have become an important approach used to understand the adverse effects of toxicants exposure. Due to regulatory constraints and ethical considerations, the quest for alternatives to animal testing has become highly recommended (MacGregor et al., 2001). Furthermore, there are substantial activities in using *in vitro* systems to advance mechanistic understanding of toxicant activities. Therefore, *in vitro* methods are routinely used by industries and regulatory bodies in toxicity testing, safety assessment, and risk evaluation, and offer unique advantages (more time and cost-effective than in vivo toxicology). The greatest use of *in vitro* methods, however, is for elucidating mechanisms of toxicity and/or demonstrating the biological process involved in response to xenobiotics (Eisenbrand et al., 2002).

Moreover, improved cell culture technologies for human cell lines and tissues can play an important role in improving the scientific bases for toxicological risk assessment. In fact, the use of these human *in vitro* systems in risk assessments not only can reduce the use of animals in toxicology studies but also helps to decrease the uncertainties of extrapolating animal toxicity data to humans as endpoints.

As example, the Caco-2 cell line, developed from an intestinal carcinoma, has been proven to be a valuable model for the study of mechanisms of local and systemic toxicity (MacGregor et al. 2001). These intestinal cells represent an important line of defense against oral administrated contaminants, eventually susceptible contaminated water, and thus, it could be a good *in vitro* model for toxicological and risk assessment studies.

7. Objectives and thesis outlines

The aims of this study are, firstly, to shed some light on the LAS and anionic surfactants water contamination in arid and semi-arid regions (Sfax, Tunisia as example) through anionic surfactants monitoring in different wastewater samples, and wastewater treatment optimization for anionic surfactants removal; And secondly, to investigate the toxicological effects of LAS, one of the major anionic surfactants ($C_{12\ h25}C_{6\ h4}SO_3Na$) on human intestinal Caco-2 cells, as an *in vitro* cell model, in order to understand the underlying mechanisms behind these effects and identify appropriate informative molecular biomarkers reflecting the associated mode of actions. Moreover, the author attempted to apply and investigate the sensitivity of the identified biomarkers in the risk assessment of wastewater samples obtained from arid/semiarid lands in Tunisia, which provided perspectives and insights into the future application of such biomarkers.

This work is presented in five chapters and the outline of the dissertation is summarized in **Fig. 1. 2.**: The first chapter introduces water major problems in arid and semiarid regions and emphasis on the importance of improving the risk assessment process, as a major step towards wise management and good decisions, through incorporating biomarkers and usage of *in vitro* systems and techniques. In the second chapter, we tried to emphasize on the relevance of anionic surfactants water contamination, and proposed to optimize a new method for the treatment of an industrial wastewater, highly loaded with anionic surfactants, emerging from a detergent/cosmetic industry in Sfax city in Tunisia. The third chapter investigates the adverse effects of LAS, one of the major anionic surfactants found on the market, on Human intestinal Caco-2 cells at different concentration panels, and gives insights into the underlying mechanisms. Chapter four aims to identify appropriate biomarkers associated with LAS cytotoxic effect in Caco-2 cells at relatively high concentrations, and LAS-induced potential

tumor promotion effect at relatively low non-cytotoxic concentrations. Furthermore, the sensitivity of the identified biomarkers was tested and applied in the risk assessment of wastewater samples from an industrial area in Sfax, Tunisia. Finally, in the fifth chapter, the main conclusions and perspectives of this work are presented.

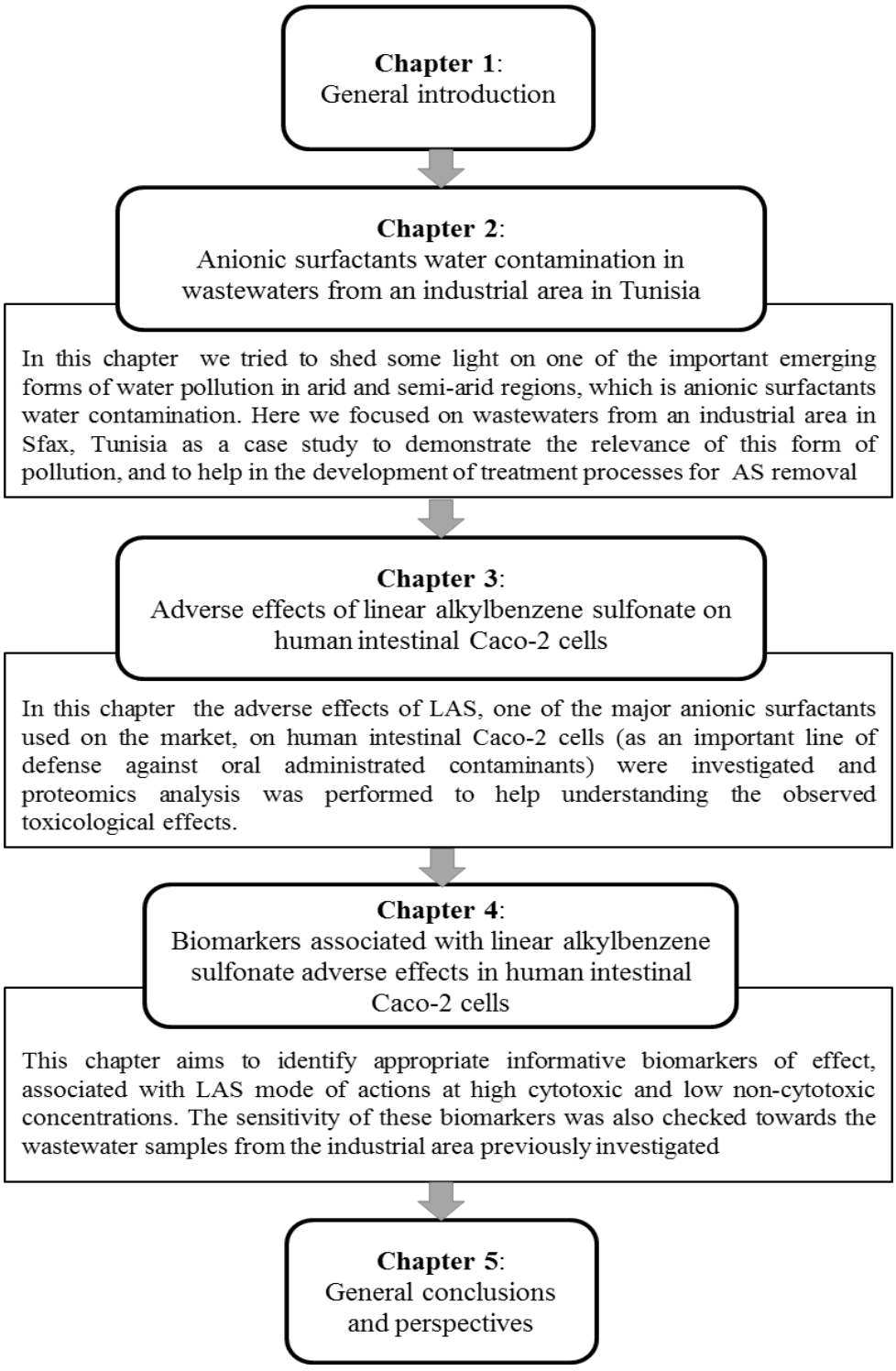


Fig. 1. 2. Outline of dissertation

CHAPTER 2

Anionic surfactants water contamination in wastewaters from an industrial area in Tunisia

1. Introduction

In the arid and the semi-arid region, countries like Tunisia are facing increasingly more serious water shortage problems. Problems of water scarcity will intensify because of population growth, rise in living standards, and accelerated urbanization which threaten the water supply in general and lead to both an increase in water consumption and pollution of water resources (Maelet and Ruelle, 2002). Continuing increase in demand by the urban sector has led to increased utilization of fresh water for domestic purposes, on the one hand, and production of greater volumes of wastewater on the other. One way to cope with these problems is to reuse wastewater. This solution requires developing new methods and technologies for the treatment of polluted water.

In Tunisia, anionic surfactants are very common pollutants found in water, with more than 100000 m^3/year of surfactant containing wastewater released, coming mainly from detergent and cosmetic industries as well as daily uses such as washing and cleaning (Dhouib et al., 2005).

In this chapter, we focused on the relevance of anionic surfactants water contamination in an example of semiarid regions: Thyna, Sfax, Tunisia. We explored different wastewaters from the industrial zone in that area and checked their levels of anionic surfactants. Also we tried to optimize a wastewater treatment process for anionic surfactants removal by integrating a physic-chemical pretreatment coupled with a membrane bioreactor.

2. Materials and methods

2.1. Wastewater samples

The wastewater samples used in this study were collected from different points in Thyna area in Sfax city, Tunisia. The samples includes: WWTP Effluent of a cosmetic industy rich in anionic

surfactants (Cos), effluent discharge of the industrial area Thyna (Mixture of several industrial effluents) (Ind), the Inlet (influent) of the WWTP Sfax south (urban wastewater + industrial wastewater) (Inlet) and the outlet (effluent) of the WWTP Sfax south. A primary filtration using a 0.45 µm filter was carried out on the sampling day. Samples were stored at -20 °C until transport inside icebox to the laboratory where the same conditions were maintained. A secondary filtration using a 0.22 µm filter was performed prior to use in the bioassays following pH adjustment.

2.2. Cell viability

Cell viability was assessed using the conventional MTT reduction assay. The cultured Caco-2 cells in 96-well plates were treated with different concentrations of wastewater samples for 24 h and 48 h, then 10 µl of MTT stock solution (5 mg/ml) was added to the culture medium and incubated for 6 h at 37 °C. The formazan was dissolved in 100 µl 10% SDS (W/V) and the absorbance was measured using a microliter plate reader at 570 nm wavelength. Cell viability was determined as the mean of absorbance at 570 nm and expressed as the percentage of the control.

2.3. Wastewater characterization: Analytical techniques

The wastewater was collected from the equalization tank of wastewater treatment plant of a cosmetic company (HENKEL, Sfax, Tunisia) during the optimization, characterized and stored at 4°C. The characteristics are shown on **Table 2. 6.**.

- pH was analyzed using a Metrohm pH-meter.

- Methylene Blue Active Substance (MBAS) assay used to the estimation of anionic surfactants. This assay was carried out according to Tunisian Norms NT 01-28 (1983). As well, Hyamine colorimetric method was also used for estimating the anionic surfactants in wastewater when concentrations exceed 40 mg/l.

- COD was estimated as described by Knechtel (1978).

- Total suspended solids (TSS) were measured as mentioned in the standard methods for examination of water and wastewater (American Public Health Association / American Water Works Association / Water Environment Federation, 1992).

2.4. Coagulation-flocculation

Flocculation and coagulation are mainly used, when the application of sedimentation is not feasible, due to the presence of extremely fine particles or globules, which do not possess a significant settling rate, because the phases do not appreciably differ in density from the parent liquid (Zouboulis and Avranas, 2000).

The experiments were carried out at laboratory bench scale using a jar test apparatus "Numerical Flocculator 10408, Fisher Bioblock Scientific" with alumina sulfate and lime as described by Aloui et al. (2009).

2.5. Optimization experimental design

The central composite design (CCD), which is the standard RSM, was selected to optimize two most effective operating variables in the coagulation–flocculation process, namely the coagulant

dosage and pH. For statistical calculations, the variables X_i were coded as x_i according to the following equation: $x_i = \frac{X_i - X_0}{\delta X}$

Where X_i is the uncoded value of the i^{th} independent variable, X_0 the value of X_i at the centre point of the investigated area and δX is the step change. The range and levels of pH (X_1) and Coagulant dosage (X_2) are given in **Table 2. 7.**. The two responses measured, COD removal (Y_1) and AS removal (Y_2), were calculated in %, and the response variables were fitted by a second-order model in the form of quadratic polynomial equation:

$$Y_m = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i}^{i<j} \sum_{j} b_{ij} X_i X_j \qquad (2)$$

Where Y_m is the response variable to be modeled; X_i and X_j the independent variables which influence Y_m; b_0 , b_i , b_{ii} and b_{ij} are the offset terms, the i^{th} linear coefficient, the quadratic coefficient and the ij^{th} interaction coefficient, respectively.

Model terms were selected or rejected based on the P value (probability) with 95% confidence level. Three additional experiments were conducted to verify the validity of the statistical experimental models (test points method) (Run No. 17, 18 and 19 in **Table 2. 8.**).

2.6. Statistical analysis

Data are presented as means ±SD. Statistical analyses of changes, for each time and concentration point compared to control, were performed using a paired two-tailed Student's t-test. A *p*-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Anionic surfactants monitoring in different wastewaters from an industrial area with a cosmetic detergent factory.

To study anionic surfactants water contamination in arid and semiarid regions, we curried out sampling from different wastewaters in an industrial area in Sfax, Tunisia, which are likely to contain high anionic surfactants levels due to the presence of a detergents/cosmetics factory in this area. The samples are labelled as follow (**Fig. 2. 1.**):

Cos: Wastewater treatment plan effluent of the detergents/cosmetics factory.

Ind: Effluent discharge of the whole industrial area Thyna (Mixture of several industrial effluents).

Inlet: The Inlet (influent) of the wastewater treatment plant of Sfax south (treating both urban wastewater + industrial wastewater).

Outlet: The outlet (effluent) of the wastewater treatment plant of Sfax South.

To get a closer look on the sampling site, the sampling points are represented by stars on **Fig. 2. 1.**. And as it is shown, the **Cos** sample is derived from the effluent of the detergents/cosmetics factory; the **Ind** sample regroups wastewaters from the whole industrial area; **Inlet** sample regroups wastewaters from both urban and industrial areas; And **Outlet** sample is the effluent of the wastewater treatment plant of Sfax South.

These samples were analyzed for their anionic surfactants contents and from the results shown in **Table 2. 1.**, we can clearly notice that the wastewater samples contain relatively high levels of anionic surfactants, largely exceed the standards specified by law for the release in public sewage (5 mg/l) for **Cos**, **Ind** and **Inlet**; and water environments (less than 1 mg/l) for **Outlet**.

The cytotoxicity of these samples on Caco-2 cells was also studied at different concentrations and treatment times, and MTT assay results (**Fig. 2. 2.** and **Fig. 2. 3.**) revealed different toxicity behaviors between the four wastewater samples on Caco-2 cells: **Cos** and **Ind** samples seem to be the most cytotoxic for both treatment times and concentrations. The **Inlet** and **Outlet** samples showed slight but significant cytotoxicity only after 48 h of treatment.

The high cytotoxicity observed for **Cos** and **Ind** samples can partly be explained by the presence of some toxic compounds in their composition, such as Phtalazinone and Trimethylpentane, as revealed by GC/MS/MS analysis (**Table 2. 2.**, **Table 2. 3.**, **Table 2. 4.** and **Table 2. 5.**)

3.2. Cosmetic/detergent wastewater treatment optimization for anionic surfactant removal

MBR technology has shown very good performance for the treatment of this kind of pollutants (Dhouib et al., 2005). But this method is confronted to some limits in high concentration of AS, due to their toxicity toward microorganisms and foaming in aerated bioreactors, on the hand, and high COD and Total Suspended Solids (TSS) which accelerate the clogging of the membrane of the bioreactor, in the other hand; which made the idea of integrating a physico- chemical pretreatment, interesting to overcome these limits.

In this work, we tried to optimize a new method for the treatment of an industrial wastewater, highly loaded with anionic surfactants (about 4 g/l), especially Linear Alkyl Sulfonate (LAS), coming from a cosmetic industry in Sfax city in Tunisia. This method combines a physicochemical pretreatment by coagulation-flocculation using lime and alumina sulfate, with a biological treatment using MBR (**Fig. 2. 4.**). The optimization was performed on the operational parameters of the new physicochemical pretreatment to reduce the COD and the anionic

surfactant amount (AS) by, at least, 30% and 25% respectively, in order to allow the good operating of the MBR, and thus, the release of an outlet that fit the standard NT106.02.

* Modeling of COD removal (Y_1):

The COD removal values of the coagulation–flocculation experiments are listed in **Table 2. 8.**. The following equation is a regression model with the experimental results:

$$Y_1 = 24.547 - 0.974X_1 + 9.557X_2 - 2.966X_1^2 + 3.119X_2^2 - 5.503X_1X_2 \quad (3)$$

Statistical testing of the model was performed with the Fisher's statistical test for analysis of variance (ANOVA) (**Table 2. 9.**). The quadratic regression shows that the model was significant and the p-value (0.0238) implies that the second-order polynomial model fitted the experimental results well. The contour plots of Y_1 (**Fig. 2. 5. a.**), obtained from the model, show that the latter has an hyperbolic shape describing the double effects of the factors interaction. The COD removal surface, presented in **Fig. 2. 5. b.**, gives an idea about the coordinate of the desired COD removal percentage, serving for the multiple responses optimization.

* Modeling of AS removal (Y_2):

The following model was obtained from the analysis of AS removal results (**Table 2. 8.**):

$$Y_2 = 5.533 - 7.373X_1 + 11.330X_2 + 4.608X_1^2 + 9.732X_2^2 - 2.687X_1X_2 \quad (4)$$

The ANOVA analysis (**Table 2. 10.**) shows that the model was significantly valid (p-value<0.001).

The contour plots (**Fig. 2. 6. a.**) show that the AS removal model has an elliptic shape proving existing interactions between factors effects, these plots have allowed to draw the corresponding response surface (**Fig. 2. 6. b.**), showing different response levels ranging from non-significant removal, about 0.5 to more than 48 %.

* Multiple responses optimization:

As shown in the contour plots, the two responses have two different behavior in the experimental range, hyperbolic for the COD removal and elliptic for the AS removal, and the optimal conditions for the two responses cannot match. Therefore, to obtain a common optimum for the two responses at the same time, we chose to use the desirability functions. The partial desirability functions D1 (for COD removal) and D2 (for AS removal) were set as follow (**Fig. 2. 7.**): For D1 satisfaction start from at least 30% of removal which is required for the MBR smooth functioning, and achieves 100% for 50% of removal, which allow a good operating even with an unexpected increase of pollution loads. The same for D2, except the satisfaction start from 25% for the same reason.

$$D = (D_1 * D_2)^{1/2} \qquad (5)$$

The global desirability function (eq. (5))was maximized and we obtained an optimal global desirability of 85.57% with 83.7% for D1 and 87.48% for D2. This optimum correspond to coded pH level of -0.7328, and coded coagulant dosage of 1.2819, which match with the real values: pH of 7.02 and coagulant dosage of 4.011g/l.

The optimized conditions were applied on the industrial process, and the results in **Table 2. 11.** were obtained from several trials and compared to the Tunisian standards. We note that the outlet

of the whole process fit the standard in the AS concentration allowed, as well as in the other parameters.

The integration and the optimization of the physicochemical pretreatment has allowed the good operating of the treatment process and the release of a treated surfactant containing wastewater that fit the standards specified by Tunisian law.

4. Conclusions

All the identified compounds (GC/MS/MS) are well chemically characterized; however, many of them do not have any available toxicological data (unknown adverse effects). Therefore, despite the huge efforts done in the investigation of chemicals toxicological effects, the progress in this field still too much insufficient and more efforts are needed.

Moreover, as shown from our example (Industrial area in Sfax, Tunisia) Wastewaters in Arid /semi-arid regions may contain relatively high concentrations of anionic surfactants that in many cases largely exceed the regulation. Thus, it is very important to study the toxicological effects of these pollutants and their risk assessment in the water environments.

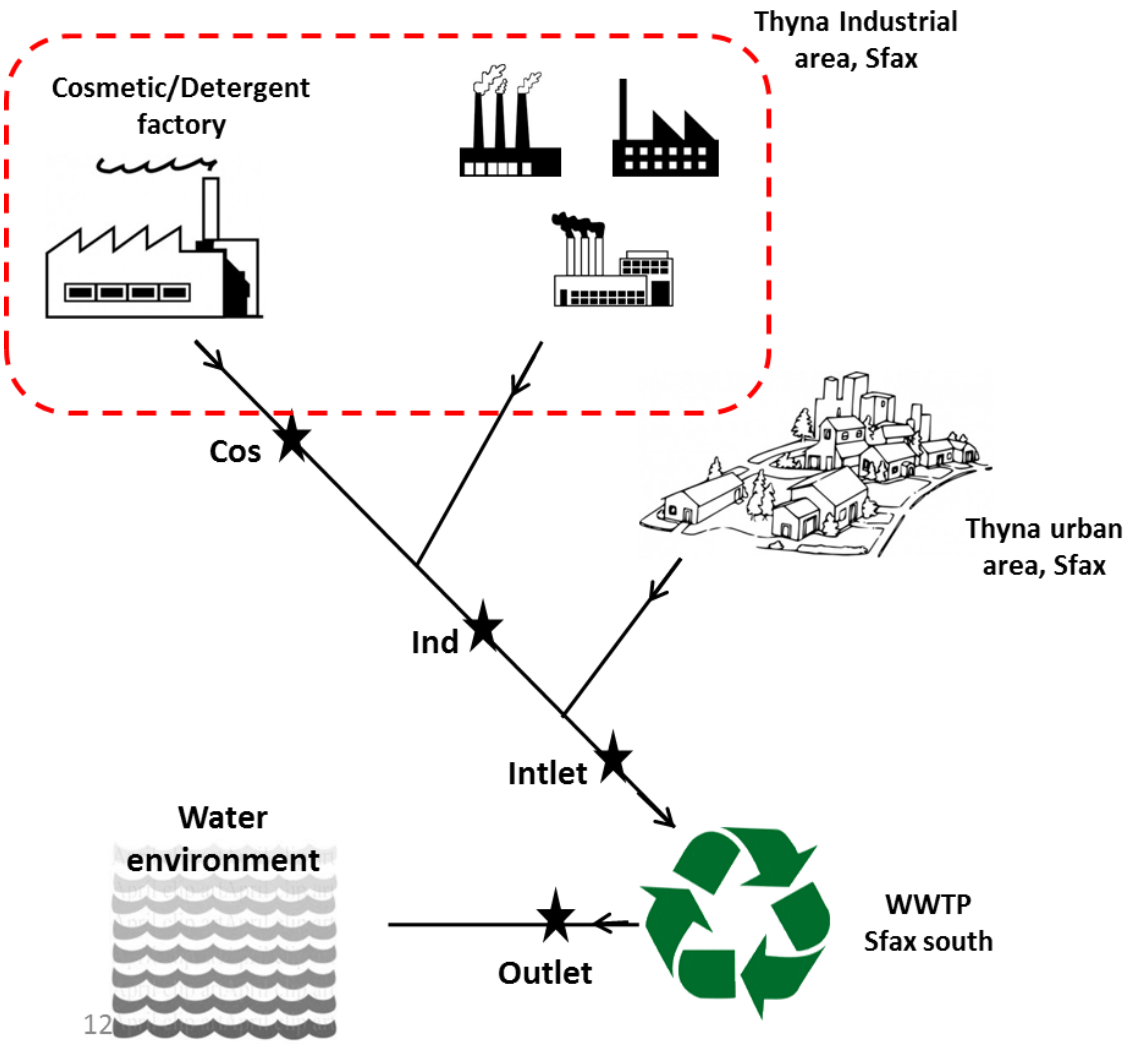


Fig. 2. 1. Sampling site of wastewater samples: Thyna industrial area, Sfax, Tunisia

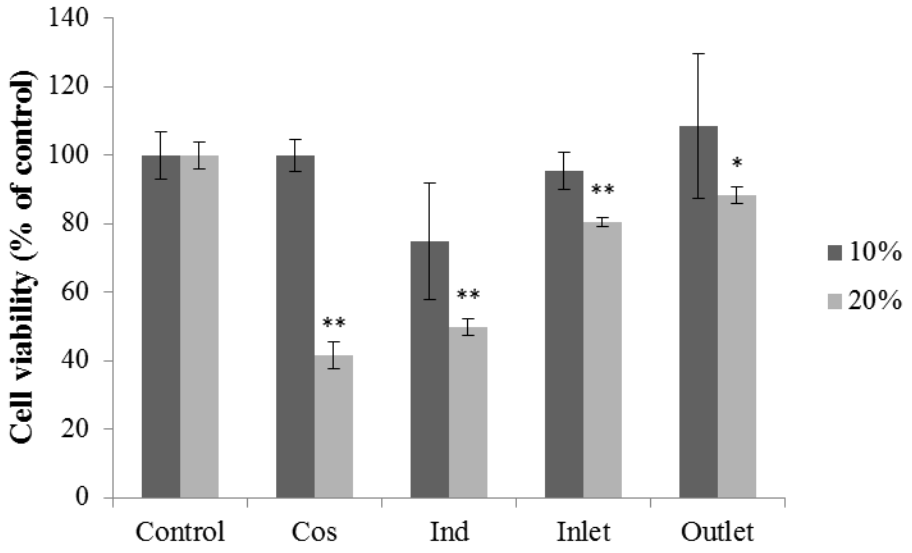


Fig. 2. 2. Reduction of MTT by Caco-2 cells after exposure to concentrations of 10% and 20% of different wastewater samples: Cos (Cosmetic wastewater), Ind (mixture of industrial wastewaters), and the inlet and outlet of southern Sfax municipal wastewater treatment plant (Inlet and Outlet), for 24 h; Cell viability was determined as the mean of absorbance at 570 nm and expressed as the percentage of the control; Control represent 10% and 20% PBS(-) respectively for sample concentrations of 10 and 20%; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

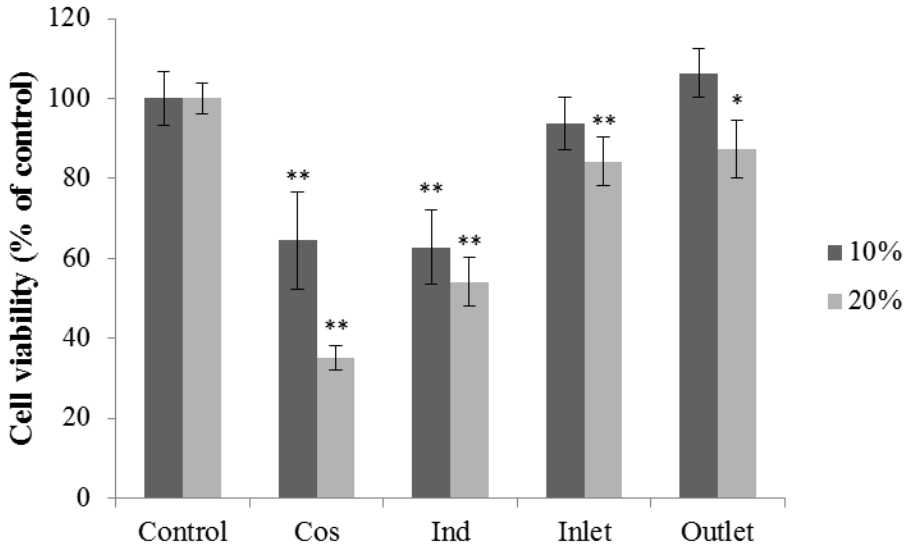


Fig. 2. 3. Reduction of MTT by Caco-2 cells after exposure to concentrations of 10% and 20% of different wastewater samples: Cos (Cosmetic wastewater), Ind (mixture of industrial wastewaters), and the inlet and outlet of southern Sfax municipal wastewater treatment plant (Inlet and Outlet), for 48 h; Cell viability was determined as the mean of absorbance at 570 nm and expressed as the percentage of the control; Control represent 10% and 20% PBS(-) respectively for sample concentrations of 10 and 20%; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

Table 2. 1. Anionic surfactants concentration in wastewater samples

Sample	Anionic surfactants (mg/l)
Cos	31.5
Ind	12.28
Inlet	30.7
Outlet	6.11

Table 2. 2. Major compounds identified in **Cos** sample using GC/MS

Compound Label	RT	Name	Formula	MFG Formula	DB Formula	Hits (DB)
Cpd 1: 3-hydroxy-4,4-dimethyldihydro(2-13C)furan-2-one	3.275	3-hydroxy-4,4-dimethyldihydro(2-13C)furan-2-one	C6 h10O3	C6 h10O3	C6 h10O3	10
Cpd 2: 3-Acetoxy-2-ethoxy-2,3,4,4a-tetrahydrodibenzofuran-3-carbonitrile	3.299	3-Acetoxy-2-ethoxy-2,3,4,4a-tetrahydrodibenzofuran-3-carbonitrile	C17H15NO4	C17H15NO4	C17H15NO4	10
Cpd 3: 2-(4-Methoxy-2,3-dimethylbut-2-enyl)-2H-1-benzothiopyran	3.313	2-(4-Methoxy-2,3-dimethylbut-2-enyl)-2H-1-benzothiopyran	C16 h20OS	C16 h20OS	C16 h20OS	8
Cpd 7: Urea, N,N-diethyl-N'-phenyl- (CAS) N,N-Diethyl-N'-phenylurea	3.409	Urea, N,N-diethyl-N'-phenyl- (CAS) N,N-Diethyl-N'-phenylurea	C11H16N2O	C11H16N2O	C11H16N2O	1
Cpd 8: 4-(3-Pyridyl)-1(2H)-phthalazinone	3.557	4-(3-Pyridyl)-1(2H)-phthalazinone	C13 h9N3O	C13 h9N3O	C13 h9N3O	10
Cpd 10: Silanamine, N-ethyl-1,1,1-trimethyl-N-(trimethylsilyl)- (CAS)	4.712	Silanamine, N-ethyl-1,1,1-trimethyl-N-(trimethylsilyl)- (CAS)	C8H23NSi2	C8H23NSi2	C8H23NSi2	7
Cpd 11: Cyclotrisiloxane, hexamethyl- (CAS) 1,1,3,3,5,5-HEXAMETHYL-CYCLOHEXASILOXANE	52.164	Cyclotrisiloxane, hexamethyl- (CAS) 1,1,3,3,5,5-HEXAMETHYL-CYCLOHEXASILOXANE	C6 h18O3Si3	C6 h18O3Si3	C6 h18O3Si3	10

Table 2. 3. Major compounds identified in **Ind** sample using GC/MS

Compound Label	RT	Name	Formula	MFG Formula	DB Formula	Hits (DB)
Cpd 2: (5S,10aS)-5-(Methoxymethyl)-1,5,10,10a-tetrahydro[1,3]oxozolo[3,4-b]isoquino...	3.313	(5S,10aS)-5-(Methoxymethyl)-1,5,10,10a-tetrahydro[1,3]oxozolo[3,4-b]isoquino...	C13 h15NO3	C13 h15NO3	C13 h15NO3	5
Cpd 3: 2-(4-Methoxy-2,3-dimethylbut-2-enyl)-2H-1-benzothiopyran	3.323	2-(4-Methoxy-2,3-dimethylbut-2-enyl)-2H-1-benzothiopyran	C16 h20OS	C16 h20OS	C16 h20OS	9
Cpd 4: Urea, N,N-diethyl-N'-phenyl- (CAS) N,N-Diethyl-N'-phenylurea	3.44	Urea, N,N-diethyl-N'-phenyl- (CAS) N,N-Diethyl-N'-phenylurea	C11H16N2O	C11H16N2O	C11H16N2O	1
Cpd 5: Trisiloxane, octamethyl- Octamethyltrisiloxane [(CH3)3SiO]2Si(CH3)2	3.583	Trisiloxane, octamethyl- Octamethyltrisiloxane [(CH3)3SiO]2Si(CH3)2	C8H24O2Si3	C8H24O2Si3	C8H24O2Si3	10
Cpd 7: 2-(trimethylsilyloxy)-2,3,4-trimethylpentane	4.742	2-(trimethylsilyloxy)-2,3,4-trimethylpentane	C11H26OSi	C11H26OSi	C11H26OSi	10

Table 2. 4. Major compounds identified in **Inlet** sample using GC/MS

Compound Label	RT	Name	Formula	MFG Formula	DB Formula	Hits (DB)
Cpd 1: S-Cyclopropyl Cyclopropanethiosulfonate	3.288	S-Cyclopropyl Cyclopropanethiosulfonate	C6 h10O2S2	C6 h10O2S2	C6 h10O2S2	4
Cpd 3: 3,3-(Ethylenedioxy)octanal 1,3-Dioxolane-2-acetaldehyde, 2-pentyl- (CAS)	3.358	3,3-(Ethylenedioxy)octanal 1,3-Dioxolane-2-acetaldehyde, 2-pentyl- (CAS)	C10H18O3	C10H18O3	C10H18O3	1
Cpd 4: 7,8,9,10-Tetrahydro-6-(2-vinylcyclopropanyl)-6(5H)-benzocyclooctenols	3.464	7,8,9,10-Tetrahydro-6-(2-vinylcyclopropanyl)-6(5H)-benzocyclooctenols	C17H22O	C17H22O	C17H22O	3
Cpd 5: Trisiloxane, octamethyl- Octamethyltrisiloxane [(CH3)3SiO]2Si(CH3)2	3.595	Trisiloxane, octamethyl- Octamethyltrisiloxane [(CH3)3SiO]2Si(CH3)2	C8H24O2Si3	C8H24O2Si3	C8H24O2Si3	10
Cpd 6: Trifluoromethyl-bis-(trimethylsilyl)methyl ketone	4.587	Trifluoromethyl-bis-(trimethylsilyl)methyl ketone	C9H19F3OSi2	C9H19F3OSi2	C9H19F3OSi2	1
Cpd 7: ETHYLAMIN, BIS-N-(TRIMETHYLSILYL)-bis-TMS Ethylamine	4.741	ETHYLAMIN, BIS-N-(TRIMETHYLSILYL)-bis-TMS Ethylamine	C8H23NSi2	C8H23NSi2	C8H23NSi2	10

Table 2. 5. Major compounds identified in **Outlet** sample using GC/MS

Compound Label	RT	Name	Formula	MFG Formula	DB Formula	Hits (DB)
Cpd 1: methyl (2R,3S,4R)-3-(tert-butyldimethylsiloxy)-2,4,5-trimethylhex-5-enoate	3.286	methyl (2R,3S,4R)-3-(tert-butyldimethylsiloxy)-2,4,5-trimethylhex-5-enoate	C16 h32O3Si	C16 h32O3Si	C16 h32O3Si	10
Cpd 2: Trideuteriomethoxycyclohexane	3.326	Trideuteriomethoxycyclohexane	C7H11D3O	C7H11D3O	C7H11D3O	1
Cpd 5: Trisiloxane, octamethyl- Octamethyltrisiloxane [(CH3)3SiO]2Si(CH3)2	3.587	Trisiloxane, octamethyl- Octamethyltrisiloxane [(CH3)3SiO]2Si(CH3)2	C8H24O2Si3	C8H24O2Si3	C8H24O2Si3	10
Cpd 6: Trifluoromethyl-bis-(trimethylsilyl)methyl ketone	4.584	Trifluoromethyl-bis-(trimethylsilyl)methyl ketone	C9H19F3OSi2	C9H19F3OSi2	C9H19F3OSi2	4
Cpd 7: ETHYLAMIN, BIS-N-(TRIMETHYLSILYL)-bis-TMS Ethylamine	4.74	ETHYLAMIN, BIS-N-(TRIMETHYLSILYL)-bis-TMS Ethylamine	C8H23NSi2	C8H23NSi2	C8H23NSi2	10

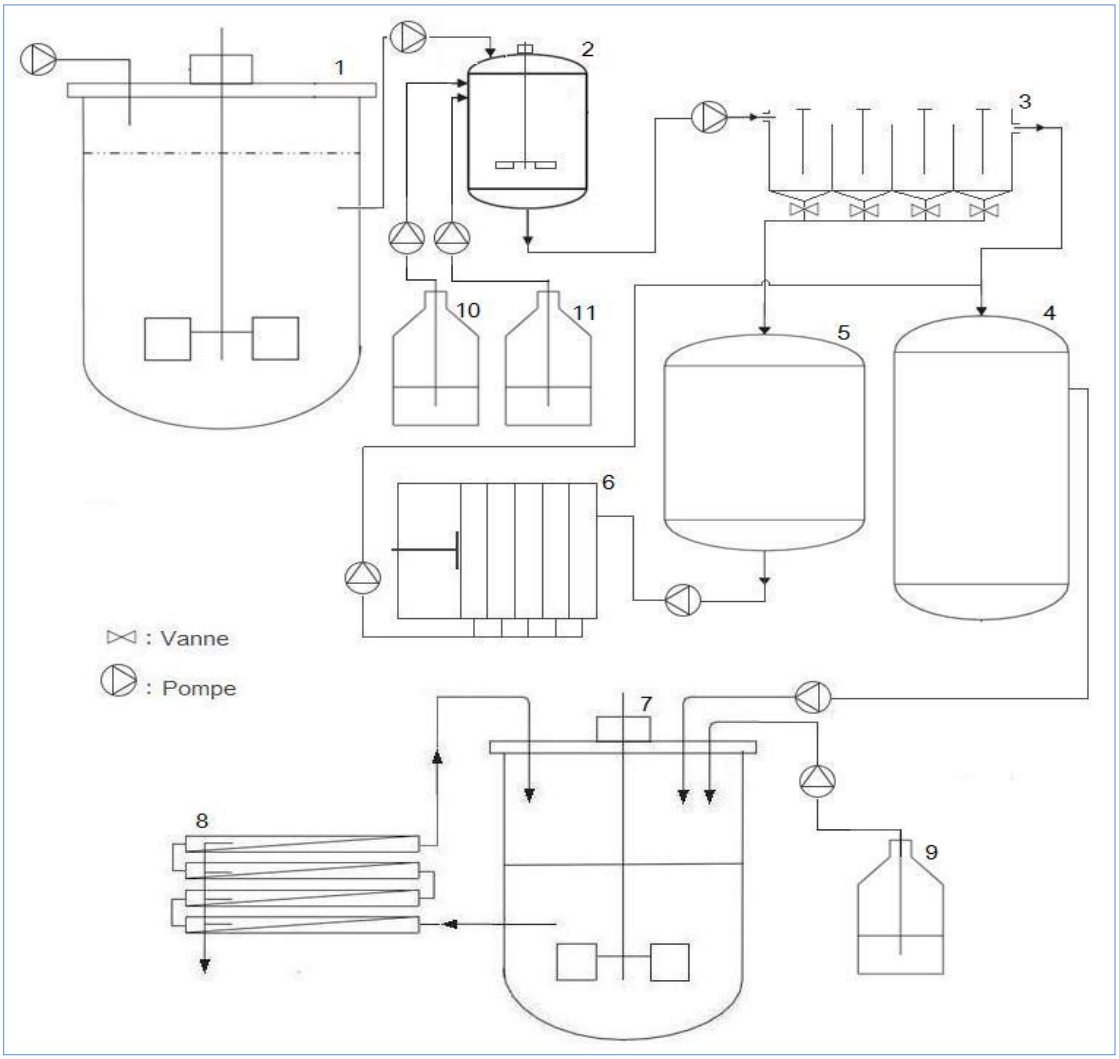


Fig 2. 4. Optimized wastewater treatment process schema. Principal treatment compartments: Collector (1), bioreactor (7), microfiltration unit (8) and ammonium nitrate tank (9); Integrated compartments for the pretreatment: Batch reactor (2), separator (3), press filter (6), chemical sludge collector (5) and filtrate tank (4).

Table 2. 6. Wastewater main characteristics

Characteristic	Mean value	Standard deviation
pH	5.56	0.32
COD (g/l)	18.250	3.112
BOD (g/l)	1.211	0.125
TSS (g/l)	2.230	0.950
AS (g/l)	3.900	0.520
N_t (g/l)	0.155	0.012

Table 2. 7. Correspondence between coded levels and real values

Coded levels (x_i)	-1.4142	-1	0	1	1.4142
pH (X_1)	6.79	7.00	7.50	8.00	8.21
Coagulant d. (X_2)	0.944	1.400	2.500	3.600	4.056

Table 2. 8. Central composite design and responses results

Run No.	Coded X_1	Coded X_2	Y_1	Y_2
1	-1	-1	8.87	24.49
2	1	-1	12.51	4.08
3	-1	1	33.49	44.90
4	1	1	16.75	16.33
5	-1.4142	0	24.63	18.37
6	1.4142	0	25.12	6.21
7	0	-1.4142	19.92	2.04
8	0	1.4142	54.76	42.86
9	0	0	23.56	8.16
10	0	0	24.08	6.12
11	0	0	34.48	8.16
12	0	0	28.10	6.12
13	0	0	24.68	4.08
14	0	0	16.47	3.04
15	0	0	23.43	3.04
16	0	0	26.61	4.16
17	-0.6124	-0.3536	27.67	6.12
18	0.6124	-0.3536	18.95	2.04
19	0	0.7071	33.36	18.37

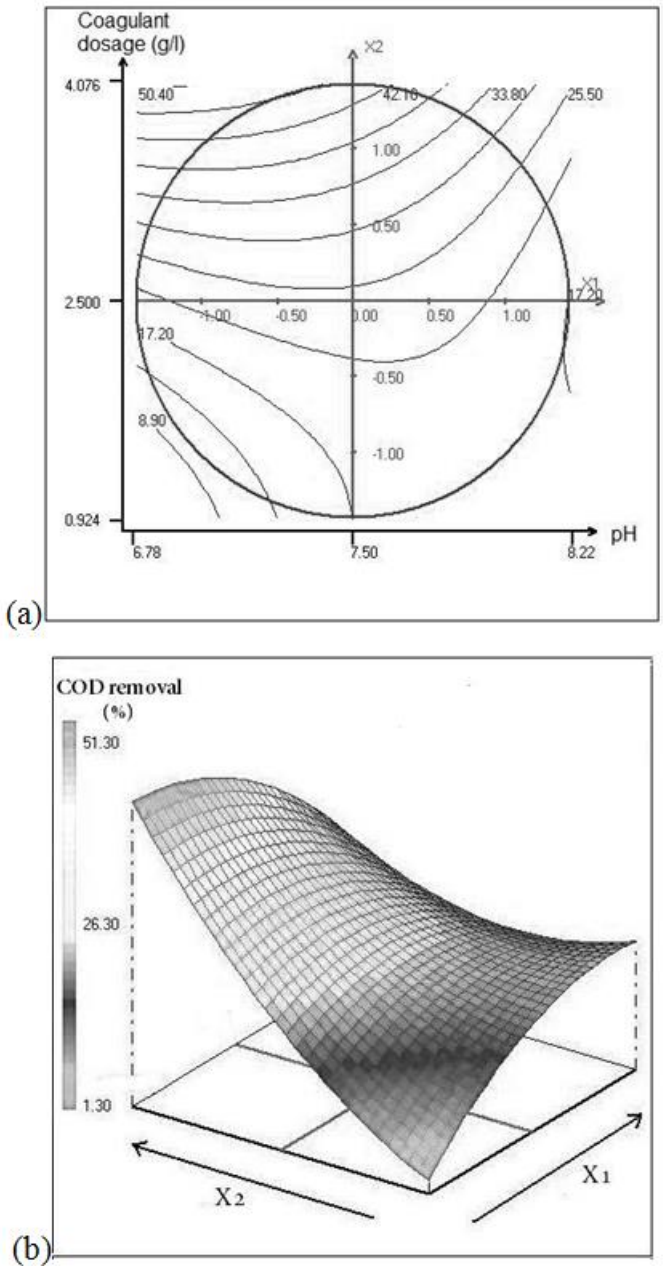


Fig 2. 5. Contour plots (a) and surface graph (b) for COD removal

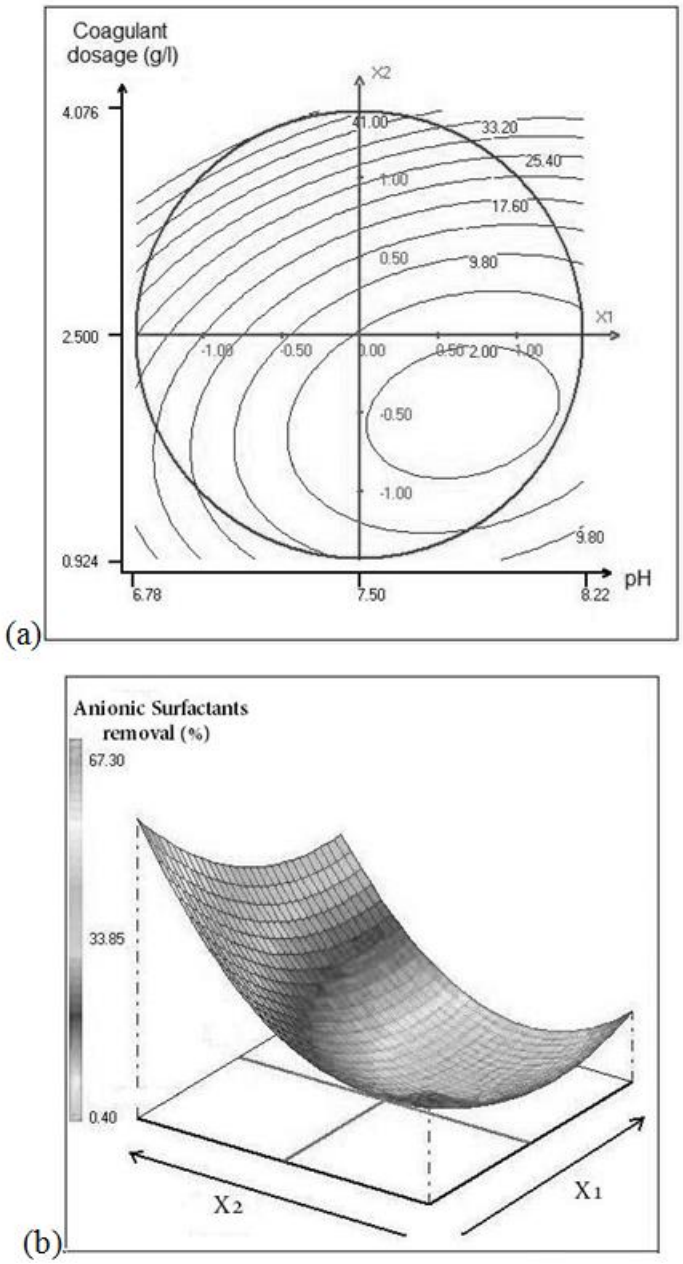


Fig. 2. 6. Contour plots (a) and surface graph (b) for AS removal

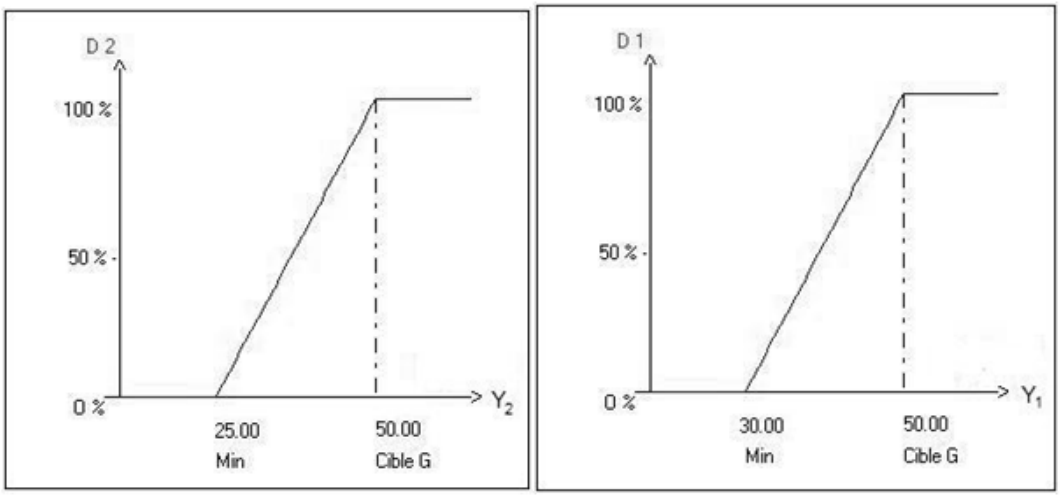


Fig. 2. 7. Partial desirability functions : D2 for AS removal(left) and D1 for COD removal (right).

Full satisfaction for each function needs at least 50% removal

Table 2. 9. ANOVA table for COD removal

Source of variance	Degrees of freedom	Sums of squares	Mean squares	F statistic	P value
Regression	1.11111E+003	5	2.22221E+002	3.8240	0.0238
Residues	7.55453E+002	13	5.81118E+001		
Validity	1.76267E+002	6	7.93778E+001	5.1488	0.0246
Error	1.07918E+002	7	1.54169E+001		
Total	1.86656E+003	18			

Table 2. 10. ANOVA table for AS removal

Source of variance	Degrees of freedom	Sumsof squares	Mean squares	F statistic	P value
Regression	2.61125E+003	5	5.22249E+002	76.3867	<0.001
Residues	3.83844E+002	13	5.95265E+001		
Validity	3.35986E+002	6	5.59976E+001	8.1905	0.0069
Error	4.78584E+001	7	6.83691E+000		
Total	2.99509E+003	18			

Table 2. 11. Industrial trials results by treatment step

Treatment Steps	COD (g/l)	AS (g/l)	pH	TSS (g/l)
Raw wastewater	19.752	4.120	5.96	3.200
Standard deviation	0.614	0.280	0.37	0.720
Pretreated ww.	11.630	2.730	7.10	0.410
Standard deviation	0.440		0.04	0.072
Inside bioreactor	3.257	0.184	6.98	8.6
Standard deviation	0.216	0.022	0.16	0.56
MBR outlet	0.824	Not detect.	7.03	Not detect.
Standard deviation	0.073	----	0.14	----
Standard NT106.02	1.000	0.005	6.50-9.00	0.400

CHAPTER 3

Adverse effects of linear alkylbenzene sulfonate on human intestinal Caco-2 cells

1. Introduction

Linear alkylbenzene sulfonates (LASs) are among the major anionic surfactants used in detergents such as, laundry powders and dishwashing products. Current estimates indicate that the total annual consumption of LASs is approximately 430,000 tons, of which nearly 350,000 tons is derived from household use as reported in HERA (2013). After use, such detergent compounds are discharged into the environment in wastewaters, and contribute to the contamination of different environmental compartments (e.g. rivers, ground water, soils). Many treatment technologies were developed for the removal of LASs in wastewater treatment plants (Beltrán-Heredia et al., 2009; de Oliveira et al., 2010; León et al., 2006). However, efficiency of these treatments still not perfect and remaining LASs can reach up to 30% (Whelana et al., 2007). Thus, the risk assessment of these compounds is of a great importance, especially that LASs are considered as toxic compounds to different terrestrial and aquatic living organisms (Debelius et al., 2008; Krogh et al., 2007; Marin et al., 1991).

Furthermore, to simplify the risk assessment approach is to consider the chemical–biological system to be a black box: that is, given an input (the chemical concentration), an output is observed (the toxicological response), with no consideration of the nature of the interaction. This approach does not provide an opportunity for tailoring the risk assessment on the basis of chemical-specific information (Clewell, 2005). Accordingly, there is a need to develop new methods integrating qualitative mode-of-action information (which elucidates the manner in which the chemical alters the biological system) in risk assessment, to help in the prediction of potential cumulative effects resulting from exposure to a mixture of contaminants having same or similar mode of actions. The development of *in vitro* cell systems in addition to the advances

in cell and tissue-based bioassays and molecular techniques, made the elucidation of toxicants mode of action not only possible, but also sophisticated.

In this part, we studied the outlines of LAS toxicological effects on human intestinal Caco-2 cells and give insight into the molecular mode of actions using Proteomics approach.

2. Materials and methods

2.1. Cell culture

Human intestinal Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 1% Penicillin/Streptomycin, and incubated in a 95% air and 5% CO_2 atmosphere at 37°C. Cell passage was carried out at a split ratio of 1:3, using 0.25% trypsin (1 mM EDTA), when cell confluence reaches 80%. All experiments were performed with cultures between passages 5 and 12.

2.2. Linear Alkylbenzene Sulfonate surfactant:

Linear Alkylbenzene Sulfonate (LAS) used in this study was purchased from Wako Pure Chemical Industries, Ltd. (Cat. No. 195-07682) as crystalline powder, having the following CAS NUMBER: 25155-30-0 , and the formula "$C_{12\ h25}C_{6\ h4}SO_3Na$". LAS solutions used for all experiments were prepared in DMEM medium already set for Caco-2 cell culture.

2.3. Cell viability

Cell viability was assessed using the conventional MTT reduction assay. The cultured Caco-2 cells in 96-well plates were treated with different concentrations of LAS for 24 h, 48 h and 72 h,

then 10 µl of MTT stock solution (5 mg/ml) was added to the culture medium and incubated for 6 h at 37 °C. The formazan was dissolved in 100 µl 10% SDS (W/V) and the absorbance was measured using a microliter plate reader at 570 nm wavelength. Cell viability was determined as the mean of absorbance at 570 nm and expressed as the percentage of the control.

2.4. Proteomics

- Protein extraction and quantification

Caco-2 cells were seeded at $2x10^5$ cells/ml density in Petri dishes. After 24 h, cells in treatment dishes were treated with 60 ppm LAS for 24 h, while control cells were not treated. Then total proteins were extracted in lysis buffer as described by Tsolmon et al. (2011), and after centrifugation, supernatant was assayed for protein content by Plus One 2D Quant kit (GE Healthcare) following the manufacturer's instructions, then stored at -80°C.

- Two-dimensional gel electrophoresis (2-DE)

First dimension was carried out using the Ettan IPGphor II (Amersham Biosciences) as previously described by Tsolmon et al. (2011). Briefly, samples containing equal quantities of protein (400 mg) were diluted to 350 ml with rehydration buffer, then subjected to Isoelectric focusing (IEF) after overnight rehydration on Immobiline DryStrips (24 cm; pH 3–10 linear). Focusing was performed as follows: 1 h at 500 V, 1 h at 1000 V, 3 h at 10000 V and 2 h 45 min at 10000 V for a total of 56 kVh. After equilibration with DTT and iodoacetamide-containing buffer, strips were transferred onto 10% vertical slab gels and SDS-PAGE was run at 280 Wh on EttanDaltSix electrophoresis system from Amersham Biosciences.

- 2-DE gels image analysis

Separated proteins were stained with Coomassie R-350 stain (GE Healthcare) in 30% methanol and 10% acetic acid. Gels were scanned at 600 dpi resolution and differential spot expression was performed using ImageMaster^{TM} 2D Plati-num 5.0 software (Amersham Biosciences). After automatic spot detection and matching, the authenticity and outline of each spot was validated by eyes and edited manually where necessary. The pairs were labeled with annotations that define tie points for gel matching. Then, after normalization to the volume of all spots in the gel, the normalized volume for each spot in the control gel was compared to the normalized volume of matched spot in the treated samples gels.

- In-gel digestion and mass spectrometry

As previously described by Tsolmon et al. (2011), Spots were excised and treated with the destaining solution. Gel pieces were washed in 100 mL ACN for 5min and briefly dried at room temperature followed by reduction with 10 mM DTT in 25 mM NH_4HCO_3 and alkylation with 55 mM iodoacetamide in 25 mM NH_4HCO_3. Spots were digested overnight by 10 mg/mL trypsin in 50 mM NH_4HCO_3 at 37°C. Ten microliters of peptide mixtures were analyzed by on-line capillary UltiMate 3000 proteomics MDLC system (Dionex) coupled to a nanospray 3200QTrap MS/MS system (Applied Biosystems). The obtained peaks were searched using MASCOT search engine against Swiss-Prot database consisting of *Homo sapiens* sequences. The proteins with ion scores greater than 34 were significant for the Swiss-Prot database ($p< 0.05$).

2.5. Statistical analysis

Data are presented as means ±SD. Statistical analyses of changes, for each time and concentration point compared to control, were performed using a paired two-tailed Student's t-test. A *p*-value < 0.05 was considered statistically significant.

3. Results and discussion

To study the adverse effects of LAS on the human intestinal aco-2 cells, the cell proliferation and viability were assessed for a wide range of concentrations (1 to 70 ppm LAS) and different exposure times (24, 48 and 72 h) using MTT assay. As shown in **Fig. 3. 1.**, LAS caused a decrease in cell viability for concentrations higher than 15 ppm for 72 h, 30 ppm for 48 h and 50 ppm for 24 h of exposure, in a time and dose dependent manner. However, when examining lower concentrations, we found that Caco-2 cell viability increased significantly, more clearly for shorter exposure time (24 h), to reach the highest level at exposure to 15 ppm LAS for 24 h.

These two different patterns observed revealed that LAS has two different effects on Caco-2 cells depending mainly on the concentration. In fact higher concentrations exert a cytotoxic behavior, unlike the relatively low concentrations which have a proliferative effect.

The Caco-2 cell line is derived from a human colon carcinoma. These cells when cultured at high confluence they differentiate to form a polarized epithelial cell monolayer, maintained by intercellular junctions such as Tight Junctions (TJ), which form a seal between adjacent epithelial cells near the apical surface, providing a physical and biochemical barrier to the passage of small molecules. This monolayer structure serves as an *in vitro* model of the human small intestinal mucosa. Exploiting this characteristic, we checked the effect of LAS on the integrity of Caco-2 monolayer. So we performed TER assay to study the permeability of Caco-2 cells monolayer. Cells were cultured in high confluence on special transwell filters until establishment of homogeneous monolayer. The established Caco-2 cells monolayer was exposed to different concentration of LAS ranging from 0 (control) to 60 ppm, and the transepithelial electrical resistance was measured continuously for 3 h.

The TER assay results presented in **Fig. 3. 2.** show a decrease in the transepithelial electrical resistance of the Caco-2 cells monolayer from the first 30 min of exposure to different concentrations of LAS in a dose dependent manner. Which signify a decrease in the barrier function efficiency through TJ disruption. This observed decrease in the efficiency of the barrier function should catch a great attention, since this suggest that the presence of LAS together with other toxicants in a co-exposure scenarios will increase the paracellular permeability of the epithelial intestinal monolayer to these toxicants and allow them to be in direct contact with more internal tissues increasing consequently the associated hazard.

To get an insight into the underlying mechanisms of LAS adverse effects on Caco-2 cells proteomics approach was utilized. Classical methods such as using specific antibodies to detect known proteins by immunolabeling or observation of changes in gene expression for specific mRNAs by PCRs have some limitation due to their difficulty in identifying proteins with no previously described function or implication in cell response to a particular toxicant. Thus, to identify molecular actors associated with the cell response to LAS adverse effect, we analyzed protein expression changes occurring in Caco-2 cells exposed for 24 h to 5 ppm and 60 ppm LAS (for the proliferative and cytotoxic effects respectively). The expression patterns of protein spots appearing on CBB-stained 2-D gels were analyzed using ImageMaster^{TM} 2D platinum 5.0 software, and spots whose expressions were significantly changed in protein profiles of LAS-exposed cells versus non-exposed cells, with a percentage of volume modification exceeding 30%, were selected for identification (**Fig. 3. 3., Fig. 3. 4. and Fig. 3. 5.**). Results gave thirteen proteins of interest, which had differential expression following the exposure to LAS, compared to the non-treated cells, which may have key roles in the cell response to LAS adverse effects.

The selected protein spots were identified by LC/MS/MS on the basis of peptide mass matching with theoretical peptide masses in tryptic digests of all known proteins of human species. The identified proteins with their theoretical and observed pI and M_W values, accession numbers and scores are presented in **Table 3.**.

Different protein groups were shown to be differentially expressed in Caco-2 cells exposed to LAS: Related to cell stress, oxidative stress, calcium homeostasis disruption, cell cycle regulation, cell proliferation and cancer. We noticed that 2 main groups of proteins were identified:

-First group is proteins related to cytotoxicity and stress response.

-And the second group is proteins related to cell cycle and proliferation control, and cancer.

4. Conclusions

LAS showed two different adverse effects on Caco-2 cells depending on the exposure concentration: Cytotoxic effect at relatively high concentrations, and proliferative effect at low non-cytotoxic concentrations. LAS cytotoxicity in Caco-2 cells was demonstrated to be a non-apoptotic necrotic cell death. LAS at low non-cytotoxic concentrations induced intestinal cancer Caco-2 cells proliferation suggesting a potential tumor promotion effect. Moreover, different proteins were differentially expressed in Caco-2 cells exposed to LAS: Related to cell stress, oxidative stress, calcium homeostasis disruption, cell cycle regulation, cell proliferation, cancer.

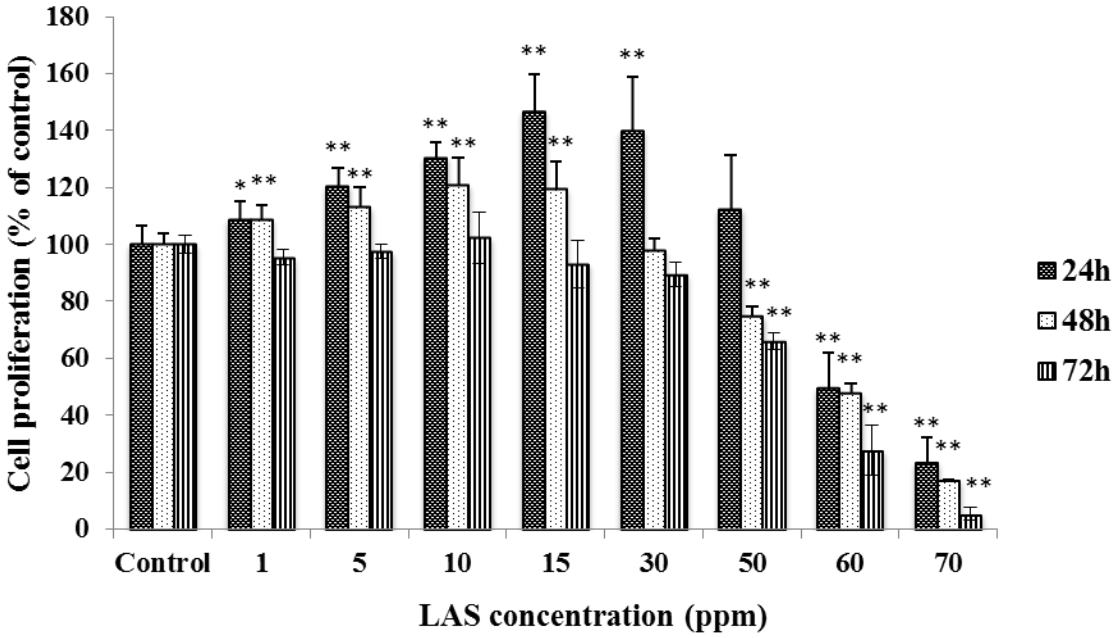


Fig. 3. 1. Reduction of MTT by Caco-2 cells after exposure to different LAS concentrations for 24 h, 48 h and 72 h; Cell viability was determined as the mean of absorbance at 570 nm and expressed as the percentage of the control; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

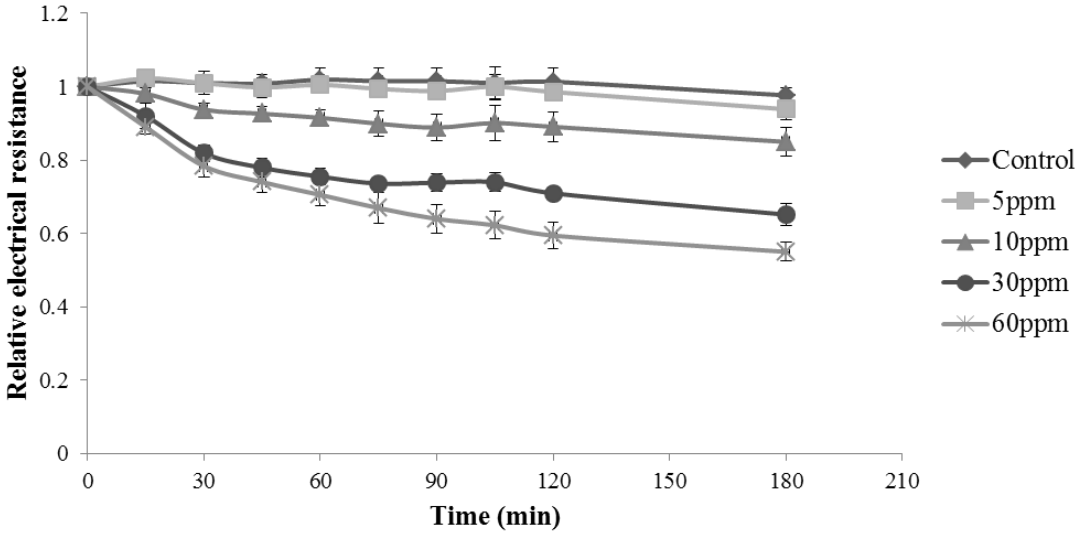


Fig. 3. 2. Effect of LAS on transepithelial electrical resistance (TER) across Caco-2 cell monolayer; Measurement was continuously conducted upon incubation with different concentrations of LAS; Results are expressed as the TER relative to that at zero time; Data represent the mean±SD of four independent experiments

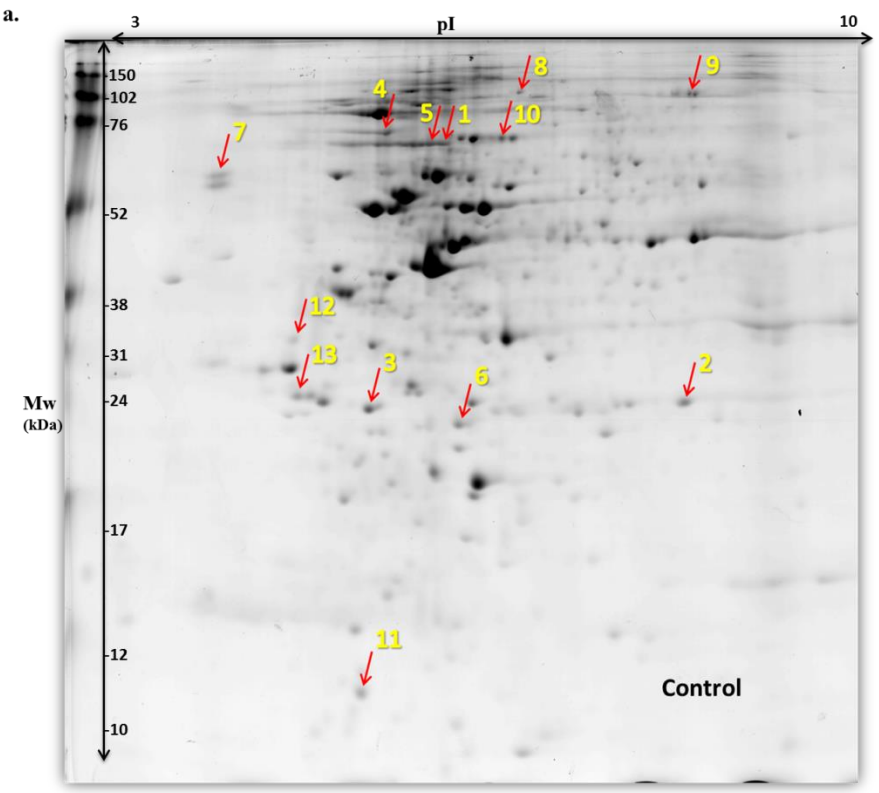


Fig. 3. 3. Representation of 2-Dimensional CBB-stained gels illustrating exemplary protein expression in Caco-2 cells with arrows indicating differentially expressed proteins after exposure for 24 h to 5 and 60 ppm LAS; (a) Control gel, (b) 60 ppm LAS treatment gel and (c) 5 ppm LAS treatment gel

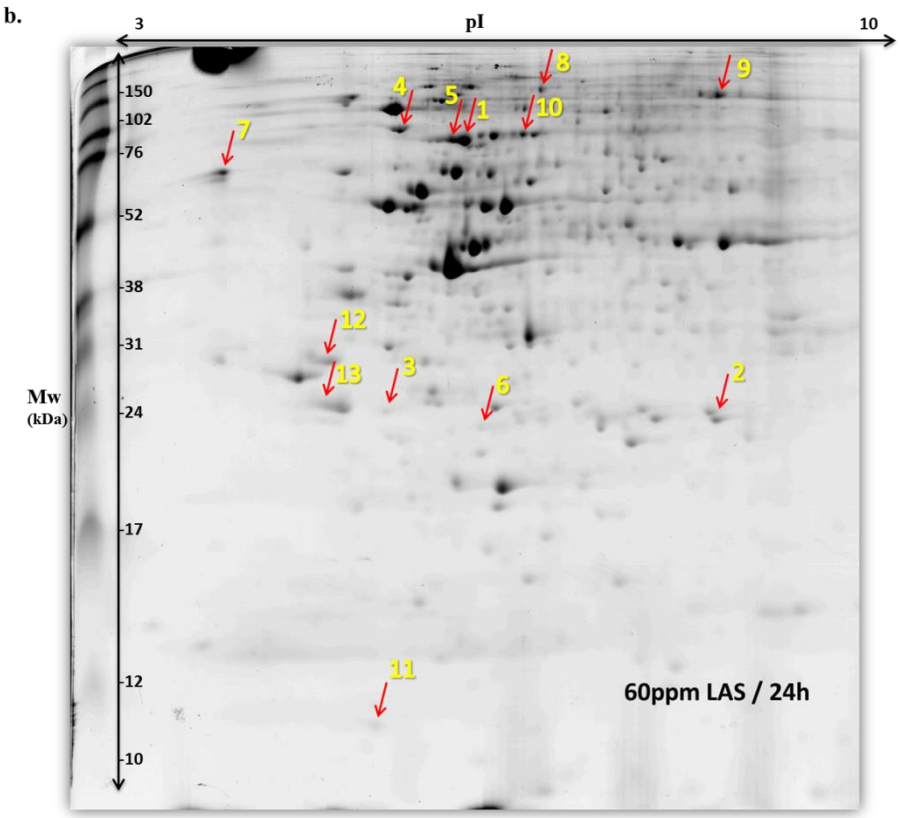


Fig. 3. 4. Representation of 2-Dimensional CBB-stained gels illustrating exemplary protein expression in Caco-2 cells with arrows indicating differentially expressed proteins after exposure for 24 h to 5 and 60 ppm LAS; (a) Control gel, (b) 60 ppm LAS treatment gel and (c) 5 ppm LAS treatment gel

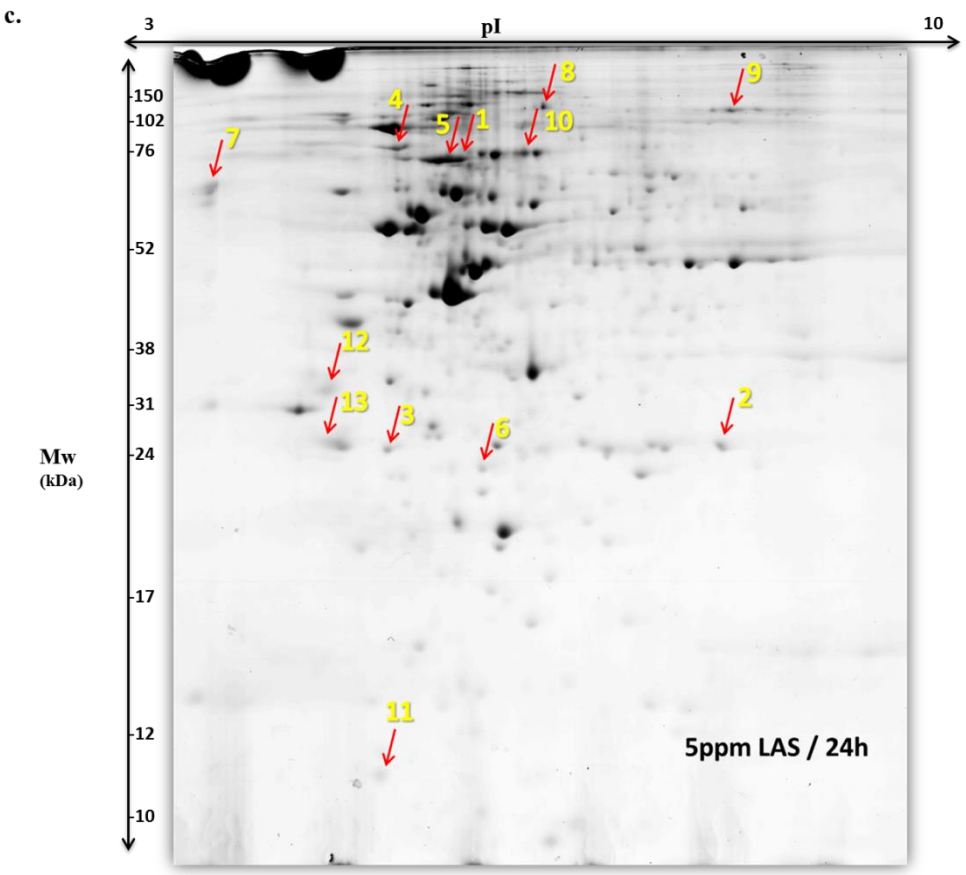


Fig. 3. 5. Representation of 2-Dimensional CBB-stained gels illustrating exemplary protein expression in Caco-2 cells with arrows indicating differentially expressed proteins after exposure for 24 h to 5 and 60 ppm LAS; (a) Control gel, (b) 60 ppm LAS treatment gel and (c) 5 ppm LAS treatment gel

Table 3. Summary of differentially expressed proteins in Caco-2 cells exposed to 5 and 60 ppm LAS for 24 h

Spot no.	Accession no.	Score	Calculated PI value/ Observed PI value	Calculated MW value/ Observed MW value (KDa)	Name of the Protein	Location in the cell
1	P11142	36	5.37/5.56	71/60	Heat shock cognate 71 kDa protein	Cytoplasm/ translocates to nuclei
2	P60174	221	5.65/7.15	31/22	Triosephosphate isomerase	Cytoplasm
3	Q96 hG5	178	5.29/5.10	42/23	Actin cytoplasmic 1	Cytoplasm cytoskeleton
4	Q9NY33	171	5.02/5.21	82/70	Dipeptidyl peptidase 3	Cytoplasm
5	P11142	318	5.37/5.45	71/60	HSP7C (Heat shock cognate 71 kDa protein)	Cytoplasm/ translocates to nuclei
6	P63261	195	5.31/5.74	42/20	Actin cytoplasmic 2	Cytoplasm, cytoskeleton
7	P27797	239	4.29/4.05	48/55	Calreticulin	Endoplasmic reticulum/ Cytoplasm/ cell surface
8	Q14697	101	5.74/6.03	107/130	Neutral alpha-glucosidase AB	Endoplasmic reticulum/ Golgi apparatus
9	P13639	404	6.41/7.2	96/110	Elongation factor 2	Cytoplasm

10	Q9P157	36	5.92/6.00	71/65	Serum albumin	Cytoplasm/ secreted
11	P10599	76	4.82/5.10	12/10	Thioredoxin	Cytoplasm/ nucleus
12	P06753	173	4.68/4.75	32/29	Tropomyosin alpha-3 chain	Cytoplasm, cytoskeleton
13	P27348	155	4.68/4.75	28/24	14-3-3 protein theta	Cytoplasm

CHAPTER 4

Biomarkers associated with Linear Alkylbenzene Sulfonate adverse effects in human intestinal Caco-2 cells

1. Introduction

Linear alkylbenzene sulfonates (LASs) are among the major anionic surfactants used in detergents such as, laundry powders and dishwashing products. Current estimates indicate that the total annual consumption of LASs in Europe only is approximately 430,000 tons, of which nearly 350,000 tons is derived from household use as reported in HERA (2013). After use, such detergent compounds are discharged into the environment in wastewaters, and contribute to the contamination of different environmental compartments (e.g. rivers, ground water, soils). Many treatment technologies were developed for the removal of LASs in wastewater treatment plants (Beltrán-Heredia et al., 2009; de Oliveira et al., 2010; León et al., 2006). However, efficiency of these treatments still not perfect and remaining LASs can reach up to 30% (Whelana et al., 2007). Thus, the risk assessment of these compounds is of a great importance, especially that LASs are considered as toxic compounds to different terrestrial and aquatic living organisms (Debelius et al., 2008; Krogh et al., 2007; Marin et al., 1991).

Furthermore, the concentration of the pollutant alone does not reflect necessarily its real effect on the endpoint level, especially when considering interactions and cumulative effects in environmental pollutants mixture, suggesting that the focus should be on the effect rather than the concentration for more realistic risk assessment approach. However, the current procedure for environmental/human health risk assessment is interested in the active substances taken separately. Accordingly, there is a need to develop new methods for assessing the actual risk of toxicants, which consider cumulative effects resulting from exposure to a mixture of contaminants having similar mode of actions. In the same regard, the need of including information about the mode of action is still rising to better adapt the risk assessment approach (Clewell, 2005).

Furthermore Toxicants released into the environment are generally found at concentrations much lower than those at which they may exert cytotoxicity. This is mainly due to the developments made in the treatment technologies used in the wastewater treatment plants, and the environmental regulations imposed by law. In contrast, many of these toxicants were shown to have carcinogenic or tumor promotion effects at low concentrations. As examples, glyphosate, a cytotoxic organophosphate herbicide, showed a tumor promoting potential in skin HaCaT cells by increasing cell proliferation and preventing apoptosis at low concentrations (George and Shukla, 2013). Also, it has been reported that exposure to low-concentration arsenic promotes cell proliferation and carcinogenesis both *in vitro* and *in vivo* (Wu et al., 2013). Such proliferative effect at low sub-lethal concentrations was observed for many other environmental toxicants such as phthalates, Okadaic Acid etc. (Chen and Chien, 2013; del Campo et al., 2013).

On the other hand, following the new tendency of reducing the use of animals in toxicity tests and the encouragement for the use of human cell and tissue models (MacGregor et al., 2001), in addition to the very few studies examining the adverse effects of LASs on human cell lines, we choose to carry out our study using the human intestinal Caco-2 cells which has been proven to be a valuable model for the study of cytotoxicity mechanisms (Natoli et al., 2009).

In this way, we investigated in this study the cytotoxic effect of LAS ($C_{12\ h25}C_{6\ h4}SO_3Na$) on human intestinal Caco-2 cells and we tried to understand the underlying mechanism using proteomics approach and different bioassays, in order to identify appropriate biomarkers of effect, reflecting the mode of action, which can serve for more reliable and informative future risk assessment studies.

2. Materials and methods

2.1. Cell culture

Human intestinal Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM: Sigma (D6046) containing L-Glutamine) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 1% Penicillin/Streptomycin, and incubated in a 95% air and 5% CO_2 atmosphere at 37°C. Cell passage was carried out at a split ratio of 1:3, using 0.25% trypsin (1 mM EDTA), when cell confluence reaches 80%. All experiments were performed with cultures between passages 5 and 12.

2.2. Linear Alkylbenzene Sulfonate surfactant:

Linear Alkylbenzene Sulfonate (LAS) used in this study was purchased from Wako Pure Chemical Industries, Ltd. (Cat. No. 195-07682) as crystalline powder, having the following CAS NUMBER: 25155-30-0 , and the formula "$C_{12\ h25}C_{6\ h4}SO_3Na$". LAS solutions used for all experiments were prepared in DMEM medium already set for Caco-2 cell culture.

2.3. Cell viability

Cell viability was assessed using the conventional MTT reduction assay. The cultured Caco-2 cells in 96-well plates were treated with different concentrations of LAS for 24 h, 48 h and 72 h, then 10 µl of MTT stock solution (5 mg/ml) was added to the culture medium and incubated for 6 h at 37°C. The formazan was dissolved in 100 µl 10% SDS (W/V) and the absorbance was measured using a microliter plate reader at 570 nm wavelength. Cell viability was determined as the mean of absorbance at 570 nm and expressed as the percentage of the control.

2.4. Proteomics

Protein extraction and quantification

Caco-2 cells were seeded at $2x10^5$ cells/ml density in 100 x 20 mm Petri dishes. After 24 h, cells in treatment dishes were treated with 60 ppm LAS for 24 h, while control cells were not treated. Then total proteins were extracted in lysis buffer as described by Tsolmon et al. (2011), and after centrifugation, supernatant was assayed for protein content by Plus One 2D Quant kit (GE Healthcare) following the manufacturer's instructions, then stored at -80 °C.

Two-dimensional gel electrophoresis (2-DE)

First dimension was carried out using the Ettan IPGphor II (Amersham Biosciences) as previously described by Tsolmon et al. (2011). Briefly, samples containing equal quantities of protein (400 mg) were diluted to 350 ml with rehydration buffer, then subjected to Isoelectric focusing (IEF) after overnight rehydration on Immobiline DryStrips (24 cm; pH 3–10 linear). Focusing was performed as follows: 1 h at 500 V, 1 h at 1000 V, 3 h at 10000 V and 2 h 45 min at 10000 V for a total of 56 kVh. After equilibration with DTT and iodoacetamide-containing buffer, strips were transferred onto 10% vertical slab gels and the second dimension SDS-PAGE was run at 280 Wh on EttanDaltSix electrophoresis system from Amersham Biosciences.

2-DE gels image analysis

Separated proteins were stained with Coomassie R-350 stain (GE Healthcare) in 30% methanol and 10% acetic acid. Gels were scanned at 600 dpi resolution and differential spot expression was performed using ImageMasterTM 2D Plati-num 5.0 software (Amersham Biosciences). After automatic spot detection and matching, the authenticity and outline of each spot was validated by eyes and edited manually where necessary. The pairs were labeled with annotations that define tie points for gel matching. Then, after normalization to the volume of all spots in the

gel, the normalized volume for each spot in the control gel was compared to the normalized volume of matched spot in the treated samples gels.

In-gel digestion and mass spectrometry

As previously described by Tsolmon et al. (2011), Spots were excised and treated with the destaining solution. Gel pieces were washed in 100 ml ACN for 5 min and briefly dried at room temperature followed by reduction with 10 mM DTT in 25 mM NH_4HCO_3 and alkylation with 55 mM iodoacetamide in 25 mM NH_4HCO_3. Spots were digested overnight by 10 mg/mL trypsin in 50 mM NH_4HCO_3 at 37°C. Ten microliters of peptide mixtures were analyzed by on-line capillary UltiMate 3000 proteomics MDLC system (Dionex) coupled to a nanospray 3200QTrap MS/MS system (Applied Biosystems). The obtained peaks were searched using MASCOT search engine against Swiss-Prot database consisting of *Homo sapiens* sequences. The proteins with ion scores greater than 34 were significant for the Swiss-Prot database ($p< 0.05$).

2.5. Protein expression: Western Blot

After exposure to 0 or 60 ppm LAS for different treatment times, the total proteins were extracted from Caco-2 cells using RIPA buffer (Sigma, USA) according to the manufacturer's instructions. The homogenates containing 10 μg of protein were separated by SDS–PAGE with a 10% resolving and 3% acrylamide stacking gel, and transferred to a PVDF membrane (Millipore, Billerica, MA) in a Western blot apparatus (Bio-Rad, Hercules, CA) run at 100 V for 1h. The nitrocellulose membrane was blocked with Odyssey™ blocking buffer and then probed with primary antibodies for Thioredoxin (Mouse monoclonal, Santa Cruz Biotechnology, Inc., USA), Calreticulin (Rabbit monoclonal, Abcam), HSP7C (Mouse monoclonal, Santa Cruz Biotechnology, Inc., USA) and GAPDH (Mouse monoclonal, Santa Cruz Biotechnology, Inc.,

USA). The signal was visualized using LiCor Odyssey Infrared Imaging System after reaction with goat anti-mouse IRDye 680LT (LI-COR) or goat anti-rabbit IRDye 800CW (LI-COR).

2.6. Gene expression: Real-time PCR

The expression of Calreticulin, Thioredoxin and Heat shock cognate 71 kDa protein (HSP7C), in treated Caco-2 cells, were determined by real-time PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal positive control. Primers used for these experiments were purchased from Applied Biosystems. Primers for Thioredoxin (Hs01555212_g1), Calreticulin (Hs00189032_m1), HSP7C (Hs03045200_g1) and GAPDH (Hs02758991_g1) were inventoried TaqMan® Gene Expression Assays. DNA-free total RNA of cultured cells was isolated with Isogen kit (Molecular Research Center, Japan) according to manufacturer's instructions. Briefly, cells were seeded at $2x10^5$ cells/ml and exposed to 60 ppm LAS for indicated times. Isolated RNA was ethanol precipitated, quantified and quality assessed by Nanodrop ND-1000 spec-trophotometer. About 1 mg of total RNA was submitted to reverse transcription with Superscript III (Invitrogen) using oligo(dT) primers according to manufacturer's protocol and cDNA was synthesized using thermal cycler (Applied Biosciences). cDNA amplification reactions were run on Applied Biosystems 7500/7500 fast RT-PCR system.

2.7. Intracellular ROS measurement

The determination of intracellular ROS was performed using OxiSelectTM Intracellular ROS Assay Kit, from CELL BIOLABS, INC., according to the manufacturer protocol. Caco-2 cells were cultured in clear bottom black 96-well plate for 24 h, then pre-incubated for 60 min with DCFH-DA. For treatment plates, LAS solution of 60 ppm was then introduced to the cells. Cells in control plates were not treated. H_2O_2 at 100 µM was used as positive control. After different

incubation times of 3 h, 6 h, 12 h and 24 h for LAS treatments and 1 h for H_2O_2 treatment, the DCF fluorescence was measured using plate reader at 480 nm/ 530 nm excitation/emission wavelengths and results were presented as percentage of control of DCF fluorescence intensity. Intracellular ROS was also measured in Caco-2 cells exposed to 60 ppm LAS for 24 h in presence of 1 mM of the ROS inhibitor N-Acetyl-L-Cystein.

2.8. Intracellular calcium measurement

The intracellular calcium measurement was performed using the Calcium kit II – Fluo 4 from Dojindo Laboratories Japan, following the manufacturer's protocol. Firstly, Caco-2 cells were cultured in clear bottom black 96-well plate for 24 h at an initial cell density of 105 cells/ml. Reaction solution was prepared just before use containing 50% of Quenching Buffer, 1.6% Pluronic® F127 (0.05 g/l), 1% Probenecid (250 mmol/l), 42.4% distilled water and 0.5% Fluo 4-AM (1 g/l in DMSO) (all % are of volume), then the mixture was sonicated for 1 min and mixed carefully by inverting. 100 µl of the latter solution was added to each well to sensitize cells, and the plate was incubated for 1 h at 37°C/5% CO_2. Finally, the treatment was performed with 60 ppm LAS for 3 h, 6 h, 12 h and 24 h, and with 200 µM capsaicin for 1 h as positive control (Capsaicin, a member of the vanilloid family, binds to and activates the TRPV1 (vanilloid receptor subtype 1) ion channel and induces an increase of intracellular calcium in Caco-2 cells as previously reported by Isoda et al., (2001)). Immediately after each treatment time, the fluorescence was measured at 480nm excitation / 518nm emission. The intracellular calcium was also measured in Caco-2 cells exposed to 60 ppm LAS for 24 h in presence of 1 mM N-Acetyl-L-Cystein.

2.9. Caco-2 cells monolayer permeability: TER assay

Transepithelial electrical resistance (TER) is correlated with the paracellular permeability of the cell monolayer as described by Hashimoto et al. (1997). For TER measurement, we were referred to the previously established method described by Han et al. (2005), briefly, the Caco-2 cells were grown at a density of $2x10^5$ cells/cm^2 in 6.5-mm diameter collagen coated Transwell (0.4 µm PTFE membrane) on 24-well plates. The medium was changed every 2 days, and the cells were cultured for 12 days to establish monolayer integrity. After obtaining a confluent Caco-2 cell monolayer, the cells were rinsed with PBS and the TER was then measured using a Millicell-ERS instrument (from Millipore) before and after adding various concentrations of LAS, and the results were expressed as the TER relative to that at zero time.

2.10. Cell number and cell viability

The cell number and viability were assessed using flow cytometry according to the manufacturer instructions. Briefly, Caco-2 cells were seeded at $2x10^5$ cells/ml density in Petri dishes. After 24 h, cells were treated with 5 and 15 ppm LAS for 24 h, while control cells were not treated. Then, cells were harvested, suspended in Guava ViaCount reagent and allowed to be stained for at least 5 min in darkness. The cell number and viability were measured by Guava PCA flow cytometry (Guava Technologies, CA, USA).

2.11. Cell cycle analysis

The distribution of the cell cycle phases of treated and control cells was assessed by flow cytometry. Briefly, Caco-2 cells were seeded at $2x10^5$ cells/ml density in Petri dishes. After 24 h, cells were treated with 5 and 15 ppm LAS for 24 h, while control cells were not treated. Then, cells were harvested, washed twice with PBS and fixed with 70% ethanol at 4ºC for more than 12 h. The fixed cells were then centrifuged at 500 × g for 5 min and washed with PBS twice. Cell

cycle reagent (Guava technologies) was added, and the cells were kept in darkness for 30 min at room temperature. The population of cells in each cell cycle phase was determined by a Guava PCA flow cytometry according to their DNA content.

2.12. Statistical analysis

Data are presented as means ±SD. Statistical analyses of changes, for each time and concentration point compared to control, were performed using a paired two-tailed Student's t-test. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. LAS effect on Caco-2 cells viability

In order to study the LAS cytotoxic effect in Caco-2 cells, cell viability was assessed using MTT assay for different LAS treatment concentrations ranging from 1 ppm to 70 ppm, and for different treatment times: 24 h, 48 h and 72 h. The results presented in **Fig. 4. 1.** showed that LAS exerts a time and dose dependent cytotoxicity in Caco-2 cells starting from a concentration of 50 ppm (cell viability decreased to 74.89±3.45% of control after 48 h and 74.89±3.45% of control after 72 h of exposure). The LC_{50} was determined to be 60 ppm for 24 h. For further investigation of the LAS cytotoxicity in Caco-2 cells, the LC_{50} concentration (60 ppm) was used in the following experiments.

3.2. Proteomics analysis of LAS-exposed Caco-2 cells

To get an insight into the underlying mechanism of LAS cytotoxic effect on Caco-2 cells, and to discover proteins implicated in the cell response mechanism to the cytotoxicity induced by LAS, proteomics approach was utilized. In fact, the protein expression changes occurring in Caco-2

cells were studied after 24 h of 60 ppm LAS exposure (LC_{50} concentration) versus non-exposed cells, serving as control. The expression patterns of protein spots appearing on CBB-stained 2-D gels were analyzed using ImageMasterTM 2D platinum 5.0 software, and spots whose expressions were significantly changed in protein profiles of LAS-exposed cells versus non-exposed cells, with a percentage of volume modification exceeding 30%, were selected for identification (**Fig. 4. 3.**). The selected protein spots were identified by LC/MS/MS on the basis of peptide mass matching with theoretical peptide masses in tryptic digests of all known proteins of human species. The identified proteins with their theoretical and observed pI and MW values, accession numbers and scores are presented in **Table 4. 1.**. Based on their background information, functions and implication in cell stress response and cytotoxicity, three proteins (Calreticulin, Thioredoxin and HSP7C) were selected for further investigation.

3.3. LAS effect on Calreticulin, HSP7C and Thioredoxin protein expression

In order to confirm the proteomics results, and to study the proteins levels changes of Calreticulin, Thioredoxin and HSP7C, the western blot analysis was performed for Caco-2 cells exposed to 60 ppm LAS for different treatment times, ranging from 3 h to 24 h. **Fig. 4. 5.** showed that 60 ppm LAS treatment in Caco-2 cells significantly and time dependently increased Calreticulin expression. In fact, a significant overexpression of Calreticulin was observed at short exposure time (3 h) and reached a peak of 3.11±0.86 fold of control after 24 h of exposure. Similarly, LAS-exposure significantly over-expressed Thioredoxin with a maximum effect (2.41±0.53 fold of control) observed at 12 h. Finally, for HSP7C, the results demonstrated that LAS significantly induced the protein overexpression after 12 h exposure with a maximum peak (2.23±0.12 fold of control) observed at 24 h.

3.4. LAS effect on the transcriptional activation of Calreticulin, HSP7C and Thioredoxin genes

The mRNA levels of Calreticulin, Thioredoxin and HSP7C were studied using real-time RT PCR to check their transcriptional activation in 60 ppm LAS-exposed Caco-2 cells. For this reason, the expressions of all three genes were measured at different exposure times: 3, 6, 12, 18 and 24 h. Results presented in **Fig. 4. 4.** showed a significant transcriptional activation of the Thioredoxin, Calreticulin, and HSP7C genes. Thioredoxin and Calreticulin genes'activation started from early exposure time (3 h) to reach 1.38±0.04 and 1.64±0.07 fold of control, respectively. A transient decrease in the Calreticulin transcriptional activation was noticed at 12 h treatment followed by a significant increase, after18 h. LAS-induced HSP7C gene upregulation was observed from 12 h of exposure and reached a maximum of 2.11±0.09 fold of control following 18 h incubation time.

3.5. LAS effect on ROS production and oxidative stress induction in Caco-2 cells

Since the overexpression of Thioredoxin is usually associated with the presence of oxidative stress in cells, we tried to check if the redox balance is disrupted under exposure to LAS, so we measured ROS production in non-treated Caco-2 cells (control) and in Caco-2 cells exposed to 60 ppm LAS for 3 h, 6 h, 12 h and 24 h. The results of ROS assay (**Fig. 4. 6. a.**) revealed a significant increase of ROS production in LAS-exposed Caco-2 cells, reaching about 224% of control after 3 h of exposure, and continue increasing in a time dependent manner until 24 h to reach a high level of about 357% of control. These results suggest that the exposure of Caco-2 cells to LAS at 60 ppm induces an increase in ROS production and consequently generates an

oxidative stress. The effect of LAS on ROS production at 24 h of exposure was significantly reduced in presence of 1 mM N-Acetyl-L-Cystein.

3.6. LAS effect on intracellular calcium homeostasis in Caco-2 cells

Considering that Calreticulin differentially modulates calcium uptake and release as crucial function in calcium homeostasis (Arnaudeau et al., 2002), the altered protein expression demonstrated in our results prompt us to check the intracellular calcium levels. So we measured the intracellular calcium levels in Caco-2 cells exposed to 60 ppm LAS for different exposure times: 3 h, 6 h, 12 h and 24 h. As shown in **Fig. 4. 6. b.**, the intracellular calcium level decrease significantly after 3 h to 6 h of exposure, then start increasing excessively from 12 h of exposure to reach very high levels after 24 h. The effect of LAS on the intracellular calcium increase after 24 h of exposure was significantly reduced in presence of 1 mM N-Acetyl-L-Cystein.

3.7. LAS effect on Caco-2 cells monolayer and Tight Junctions disruption

The Caco-2 cell line is derived from a human colon carcinoma. These cells when cultured at high confluence they differentiate to form a polarized epithelial cell monolayer, maintained by intercellular junctions such as Tight Junctions (TJ), which form a seal between adjacent epithelial cells near the apical surface, providing a physical and biochemical barrier to the passage of small molecules. This monolayer structure serves as an *in vitro* model of the human small intestinal mucosa.

Exploiting this characteristic, we checked the effect of LAS on the integrity of Caco-2 monolayer. So we performed TER assay to study the permeability of Caco-2 cells monolayer. Cells were cultured in high confluence on special transwell filters until establishment of homogeneous monolayer. The established Caco-2 cells monolayer was exposed to different

concentration of LAS ranging from 0 (control) to 60 ppm, and the transepithelial electrical resistance was measured continuously for 3 hours.

The TER assay results show a decrease in the transepithelial electrical resistance of the Caco-2 cells monolayer from the first 30 min of exposure to different concentrations of LAS in a dose dependent manner. Which signify a decrease in the barrier function efficiency through TJ disruption.

4. Discussion

4.1. Biomarkers associated with LAS cytotoxic effect

Recent calls recommend the use of toxicants mode of action as a potential solution to improve our understanding of toxicological effects and to allow the prediction of cumulative effects resulting from combined mixture effect (Clewell, 2005). Unfortunately, this idea is encountering the problem of lack of information regarding the toxicological background of toxicants (National Academies Standing Committee on the Use of Emerging Science for Environmental Health Decisions, 2012). Furthermore, describing the outlines of the toxicological effect cannot imperatively give clear ideas about the mechanism. In fact, even for widely occurring stresses, such as oxidative stress, the cell response is very diversified depending on the cell type and the oxidant agent the cell is exposed to (Simmons et al., 2011; Staleva et al., 2004). In this regard, we intend in this study to unveil the key protein actors in Caco-2 cell response to LAS cytotoxic effect, to serve as associated biomarkers reflecting its mode of action in this human cell model. The inclusion of such informative biomarkers of effect in risk assessment process can help in the prediction of potential cumulative effects from other coexisting toxicants that may have same or similar mode of actions.

The cytotoxic effect of LAS was studied on Caco-2 cells, which represent the first investigation of LASs toxic effect on human intestinal epithelial cells and one of the few LASs toxicity studies conducted on human cell lines (Perkins et al. 1996; Hansen et al. 1997). We demonstrated, for a range of 1 to 70 ppm and exposure of 24 h, 48 h and 72 h, that LAS induced a time and dose dependent cytotoxicity in Caco-2 cells, with an LC_{50} of 60 ppm for 24 h of exposure (**Fig. 4. 1.**). It is well known that surfactants have a good performance in membrane protein solubilization inducing curvature stress that causes disordering and, finally, membrane lysis (Nazari et al., 2012). This necrotic process was also reported for LAS cytotoxicity towards different cell lines from different species such as gill epithelium cells from Terapon jarbua and testis cells of the teleostean fish (Huang et al., 1994; Trivedi et al., 2001).

Classical methods such as using specific antibodies to detect known proteins by immunolabeling or observation of changes in gene expression for specific mRNAs by PCRs have some limitation due to their difficulty in identifying proteins with no previously described function or implication in cell response to a particular toxicant. Thus, to identify appropriate biomarkers associated with the cell response to LAS cytotoxicity, we utilized proteomics approach and analyzed protein expression changes occurring in Caco-2 cells exposed for 24 h to 60 ppm LAS.

A deep analysis of the identified proteins was conducted regarding their background information, functions and implication in cell stress response and cytotoxicity, and accordingly, three proteins: Calreticulin, Thioredoxin and Heat shock cognate 71 kDa protein (HSP7C), which may have key roles in the cell response to LAS cytotoxic effect, were selected for further investigation.

Thioredoxin is a redox-regulating protein, involved in oxidative stress response via, mainly, ROS scavenging and cytoprotection functions (Watanabe et al., 2010). This protein is considered as oxidative stress marker in several diseases and infections (Jikimoto et al., 2001; Zhang et al., 2009). LAS induced the transcriptional activation of Thioredoxin at gene level from the first hours of exposure to a concentration of 60 ppm (**Fig. 4. 4.**). This observation was in accordance with results of western blot analysis showing an increased level of Thioredoxin in time dependent manner, reaching the overexpression peak after 12 h of exposure (**Fig. 4. 5.**). Additionally, we demonstrated that LAS induced a significant increase in ROS production in Caco-2 cells exposed to 60 ppm LAS (**Fig. 4. 6. a.**) from the first hours of exposure, which consequently suggest the occurrence of oxidative stress in Caco-2 cells exposed to LAS. During oxidative stress, Thioredoxin changes from reduced form to oxidized form because of its redox nature. Moreover, the 2D electrophoresis experiment assures the separation of these two forms since the pI value changes when the protein changes its redox statue (Halligan et al., 2004; Myers et al., 2008), which suggest that the identified spot on the 2D gels was relative to the reduced form of Thioredoxin, explaining thereby the observed decrease in the spot intensity after exposure to LAS (spot 3 in **Fig. 4. 3.**), while the protein level determined by western blot represents the total Thioredoxin protein. In accordance, previous studies indicated that LAS mediates its toxicological effect via induction of oxidative stress (Álvarez-Muñoz et al., 2009; Sobrino-Figueroa, 2013).

The Calreticulin is a calcium-binding chaperone involved in the regulation of intracellular Ca^{2+} homeostasis and endoplasmic reticulum Ca^{2+} storage capacity (Gelebart et al., 2005). This protein is reported to be overexpressed under oxidative stress conditions to take part in the cellular response in an antioxidant mechanism mediated by the thioredoxin up-regulation (Jia et

al., 2008). The gene expression of Calreticulin in accordance with the protein expression level (**Fig. 4. 4.** and **Fig. 4. 5.**), shows an important increase from the first 3 h of exposure. This increase, probably caused by the oxidative stress occurrence in the cell, is associated with the decrease of the intracellular calcium concentration (**Fig. 4. 6. b.**) for first hours of exposure (3 h and 6 h). Moreover, the Calreticulin overexpression in the first 3 to 6 h matches with the observed thioredoxin overexpression (**Fig. 4. 4.** and **Fig. 4. 5.**), which supports the hypothesis that the calreticulin involvement in the oxidative stress response is probably mediated by the thioredoxin regulation (Jia et al., 2008). After 12 h of exposure, intracellular calcium increased excessively to finally reach a very high level (exceeding 200% of control) for 24 h of exposure (**Fig. 4. 6. b.**). Under this significant perturbation of the intracellular calcium homeostasis, the Calreticulin level increased to reach a peak at 24 h of exposure (**Fig. 4. 4.**) after a transient decrease in the gene expression at 12 h. We explained how CALR can be involved as well in oxidative stress response, however, the overexpression of the CALR gene following the transient decrease at 12 h is probably to accomplish calcium homeostasis-related function since it is reported to be overexpressed when intracellular calcium level increases, to couple calcium release (Kwon et al., 2000). So it is probable that this change in function is accompanied by the observed transient modulation of the transcriptional activation of this gene. The high intracellular calcium level is usually associated with cell death stress (Xu et al., 2001). In a different study conducted on toat kidney epithelial cells, Bjerregaard et al. (2001) demonstrated that LAS induces increase in intracellular Ca^{2+}, and concluded that this change in ion homeostasis is thought to be the fundamental reason of the toxic effect induced by LAS. In the same regard, Yamaguchi et al. (2006) demonstrated that LAS cytotoxicity in rat thymocytes is modulated by calcium; In fact, it increases when increasing the intracellular calcium concentration.

HSP7C is a housekeeping chaperone having mainly functions related to Chaperone-mediated autophagy and prevention from proteins aggregation under stress conditions (Daugaard et al., 2007). This protein is also known to be overexpressed in some cell death stresses associated with necrosis (Basu et al., 2000). In our study, we demonstrated that LAS at cytotoxic concentration of 60 ppm induced the overexpression of HSP7C in Caco-2 cells, more significantly after 12 h of exposure reaching a peak of about 2.25 fold of control after 24 h (**Fig. 4. 5.**). The transcriptional activation of HSP7C was observed after 12 h of exposure (**Fig. 4. 4.**) which was synchronized with the increase of intracellular calcium level in the exposed cells (**Fig. 4. 6. b**.), suggesting that this activation was triggered by the new occurring stress caused by the high level of calcium in cells. Furthermore, the implication of HSP7C in necrotic cell death stress (Basu et al., 2000) comes in accordance with the reported necrotic effect of LAS (Huang et.al., 1994; Trivedi et al., 2001) suggesting the necrotic nature of LAS cytotoxicity on Caco-2 cells.

Interestingly, the effect of LAS on intracellular calcium increase was significantly reduced in presence of the ROS inhibitor N-Acetyl-L-Cystein (**Fig. 4. 6. b.**). This result suggests that LAS effect on calcium homeostasis is modulated by the ROS production and induction of oxidative stress, which implicate a succession relation between the LAS-induced ROS increase and intracellular calcium increase.

Under Oxidative stress, the barrier function of epithelial cells monolayer, basically modulated by Tight Junctions (TJ), is affected by the cytoskeleton alteration (Banan et al., 2000; Hartsock and Nelson, 2008). In fact, we demonstrated using TER assay that exposure to relevant concentration of LAS (10 to 60 ppm) induces TJ disruption and decrease the barrier function efficiency in a dose dependent manner (**Fig. 3. 2.**). This observed decrease in the efficiency of the barrier function should catch a great attention, since this suggest that the presence of LAS together with

other toxicants in a co-exposure scenarios will increase the paracellular permeability of the epithelial intestinal monolayer to these toxicants and allow them to be in direct contact with more internal tissues increasing consequently the associated hazard.

4.2. Biomarkers associated with LAS potential tumor promotion effect

The effect of LAS on the cell proliferation of human intestinal Caco-2 cells was assessed for a wide range of concentrations (1 to 70 ppm LAS) and two different exposure times (24 and 48 h) using MTT assay. As shown in **Fig. 4. 7.**, LAS caused a decrease in cell proliferation for concentrations higher than 30 ppm for 48 h and 50 ppm for 24 h of exposure. However, at lower concentrations, ranging from 1 ppm to 30 ppm, LAS significantly increased Caco-2 cell proliferation, more importantly for 24 h, to reach a maximum level of 147±13% of control at 15 ppm. The observed decrease of cell proliferation at relatively high LAS concentrations is explained by its cytotoxic effect already reported for Caco-2 cells (Bradai et al., 2014). In fact LAS exposure causes a necrotic cell death in Caco-2 cells after induction of oxidative stress followed by an excessive increase of intracellular calcium level.

Additionally, cell number and viability of 5 and 15 ppm LAS-treated Caco-2 cells were checked using flow cytometry. As shown in **Fig. 4. 8.**, total cell number increased significantly in LAS-treated Caco-2 cells compared to the control ($8.22x10^6$ $\pm0.59x10^6$ cells/ml and $1.08x10^7$ $\pm0.66x10^6$ cells/ml for 5 ppm and 15 ppm LAS respectively, compared to $5.23x10^6$ $\pm0.29x10^6$ cells/ml for control). Furthermore, a slight decrease of cell viability was noticed in cells treated with 15 ppm LAS (**Fig. 4. 8.**). This effect was also observed using Trypan blue exclusion method (**Fig. 4. 9.**) for 10 and 15 ppm LAS concentrations for 24 h, where Caco-2 cells number

increased significantly from about $1.26x10^5$ cells/ml (control) to about $1.8x10^5$ cells/ml and $2.8x10^5$ cells/ml (for 10 and 15 ppm LAS respectively).

Furthermore, the role of ROS signaling in cell proliferation and survival was highlighted in many studies proving that ROS act as positive regulators of cell proliferation (Ray et al., 2012; Sauer et al., 2001). Moreover, the role of redox balance in the control of intestinal epithelial cells proliferative activity via stimulation of proliferative responses associated with oxidative challenge was also demonstrated (Aw, 2003). Therefore, the intracellular ROS level changes were investigated in non-exposed and LAS-exposed Caco-2 cells to 5 ppm LAS for different treatment times (**Fig. 4. 10.**). Results showed a slight but significant ROS level increase in LAS-exposed Caco-2 cells after 12 and 24 h treatment times. These results suggest a potential role of the intracellular ROS small increase in the observed effect.

LAS is known to be a non-cancer inducer compound (Yam et al., 1984) and even though Caco-2 cells express estrogen receptors (Campbell-Thompson et al., 2001), LAS and its degradation products have been reported to be not estrogenic (Navas et al., 1999). Interestingly, our findings suggest that LAS at non cytotoxic concentrations and for short exposure time (24 h) can increase colon cancer cells proliferation. This effect is also known for some chemicals which are not carcinogenic (cannot initiate cancer) but have a selective effect of tumor promotion on initiated cells, such as cholic acid which is a colon cancer promoter that have effect only on aberrant colon cells (initiated cancer cells) via increasing cell proliferation (Corpet et al., 1997).

In order to investigate some possible molecular actors associated with LAS-induce proliferation in Caco-2 cells, proteomics approach was performed. In this respect, protein expression changes occurring in 5 ppm LAS treated Caco-2 cells for 24 h (concentration showing no cytotoxicity or

reduced viability (**Fig. 4. 8.**)) were studied. The expression patterns of protein spots appearing on CBB-stained 2-D gels were analyzed using ImageMaster^{TM} 2D platinum 5.0 software, and spots whose expressions were significantly changed in protein profiles of LAS-exposed cells versus non-exposed cells, with a percentage of volume modification exceeding 30%, were selected for identification (**Fig. 4. 12.**).

The selected protein spots were identified by LC/MS/MS on the basis of peptide mass matching with theoretical peptide mass in tryptic digests of all known proteins of human species. The identified proteins with their theoretical and observed pI and M_W values, accession numbers and scores are presented in **Table 4. 2.**. A deep analysis of the identified proteins was conducted regarding their background information, functions and implication in cell proliferation, cell cycle and cancer, and accordingly, three proteins: Elongation factor 2 (EF2), Dipeptidyl peptidase 3 (DPP3) and 14-3-3 protein theta (14-3-3T), were selected for further investigation.

It is well known, that in protein synthesis, the elongation step is critically governed by EF2 which is solely responsible for translocation of codons from A to P ribosome sites. The overexpression of this protein is observed in many cancers (Ovarian, gastric and colon cancers) (Alaiya et al., 1997; White-Gilbertson et al., 2009). In fact EF2 influences cell cycle progression and enhances the cell growth through the promotion of G2/M phase progression as reported by Nakamura et al. (2009) for gastrointestinal cancer cells. Our proteomics results showed that the LAS-induced increase in Caco-2 cells proliferation was associated with the overexpression of EF2 (spot 1 in **Fig. 4. 12.**) and this was confirmed at transcriptional level using real-time PCR for treatments with 5 and 15 ppm LAS for 24 h (**Fig. 4. 13.**), where the EF2 mRNA was significantly overexpressed in both treatments (3.79±0.83 and 3.37±0.28 fold of control for 5 and 15 ppm LAS treatments respectively).

Furthermore, the importance of aminopeptidases in cell proliferation has been revealed by several studies which demonstrated the growth modulatory effects of aminopeptidase inhibitors (Ino et al., 1992, 1994; Sekine et al., 2001). The aminopeptidase DPP3 has been reported to be associated with few cancers. In fact it was found to be overexpressed with increased activity in ovarian and endometrial cancers (Simaga et al., 1998; Prajapati and Chauhan, 2011), more markedly with the aggressiveness of the cancer (Simaga et al., 2003). In this study, Proteomics results revealed that LAS at concentration of 5 ppm induced the overexpression of DPP3 in Caco-2 cells after 24 h of exposure (spot 2 in **Fig. 4. 12.**). These results were confirmed at gene level in Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h (**Fig. 4. 13.**), and similar pattern for both treatments were observed (4.88±1.02 and 3.28±0.23 fold of control for 5 and 15 ppm LAS treatments respectively) which suggest the involvement of DPP3 in the LAS-induced proliferation effect on Caco-2 cells at those concentrations.

14-3-3 proteins play key roles in the regulation of central physiological pathways such as mitogenesis, cell survival signaling, cell cycle and apoptosis (van Hemert et al., 2001). They are also involved in the regulation of various oncogenes and tumor suppressor genes (Tzivion et al., 2006). Some 14-3-3 isoforms are negative regulators of cell cycle and are down-regulated in different cancers (ovarian, prostate and endometrial carcinomas) (Mhawech et al., 2005; Urano et al., 2004). 14-3-3T negatively regulates the kinase activity of PDPk1(3-phosphoinositide-dependent protein kinase 1) (Sato et al., 2002), which is crucial for cell proliferation and cell cycle progression (Nakamura et al., 2008), and an activator of NF-kappa B pathway (Lee et al., 2005) which in its turn is well recognized as central activator of the anti-apoptotic cascades (Escárcega et al., 2007). Consequently, the down-regulation of 14-3-3T has a pro-survival impact on cells and promotes cell cycle progression. In our study, LAS treatment at 5 ppm in Caco-2

cells significantly down-regulated 14-3-3T at translational level (0.44±0.12 fold of control) (spot 3 in **Fig. 4. 12.**), confirmed as well at transcriptional level (**Fig. 4. 13.**) for both 5 and 15 ppm LAS after 24 h of exposure, where gene expression decreased to 0.78±0.27 and 0.71±0.08 fold of control respectively.

Interestingly, at gene level the expression of these three proteins showed the same pattern for Caco-2 cells exposed to 60 ppm LAS for 24 h (**Fig. 4. 14.**) which has been demonstrated to be the LC_{50} concentration (**Fig. 4. 1.**). Moreover, the EF2 which is likely to be the key protein involved in the cell proliferation enhancement caused by exposure to low concentrations of LAS, can be inactivated at protein level by phosphorylation at threonine 56 as a result of the enzymatic activity of EF2 kinase. This phosphorylation can occur under different conditions as explained in **Fig. 4. 15.**. In fact at high intracellular calcium concentration the EF2 kinase become active and phosphorylation of EF2 increases resulting in the inactivation of the latter. In this regard, we demonstrated that the cytotoxicity effect of LAS at relatively high concentrations is associated with the increase of the intracellular calcium (**Fig. 4. 6. b.**). So we investigated the phosphorylation of EF2 using western blot analysis, and we demonstrated that, unlike treatments with 5 and 15 ppm LAS, Treatment with 60 ppm LAS for 24 h significantly increased the phosphorylation of EF2 (data not shown). These findings can explain in part the observed increase in EF2 gene expression at high cytotoxic LAS concentrations, since at this exposure conditions (high intracellular calcium level), EF2 is likely to be inactive through the EF2 kinase mediated phosphorylation.

Additionally, we checked the possible changes in the cell population distribution in the different phases of cell cycle of exposed-Caco-2 cells to 60 ppm LAS for 24 h. Results showed that the cell percentage in G0/G1 phase increased significantly comparing to control, while cell

population in G2/M phase significantly decreased (**Fig. 4. 16.**). These modifications in cell cycle repartition (arrest in G0/G1) are usually associated with cytotoxicity effects whether by necrosis or apoptosis for different chemical compunds (Ho et al., 2005; Pusztai et al., 1993; Sun et al., 2011).

Trying to compare both distributions of Caco-2 cells exposed to high (60 ppm) and low (5 and 15 ppm) LAS concentrations, we noticed that patterns of distribution modification were completely different. As a matter of fact, when cell percentage in S phase increases in Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h (**Fig. 4. 11.**), it remains with no significant change in to 60 ppm LAS-exposed cells. However for G0/G1 phase, while the cell population of 60 ppm LAS-exposed cells increased significantly, it remains with no significant changes for treated cells with low concentrations of 5 and 15 ppm LAS. These observations can partly explain the different patterns of LAS exposure effect at high (cytotoxic effect) and low (tumor promotion effect) concentrations in Caco-2 cells.

4.3. Sensitivity of identified biomarkers towards different wastewater samples

The transcriptional activation of previously identified biomarkers, in addition to two reference stress genes HSP70 and HSP90, was assessed for Caco-2 cells exposed to 10% concentration of wastewater samples. Cytotoxicity biomarkers: THIO, CALR and HSP7C (**Fig. 4. 17.**) were significantly overexpressed in all wastewater samples but with different patterns even in samples with no cytotoxicity for 24 h (**Fig. 2. 2.**). CALR biomarker of calcium homeostasis disruption was best overexpressed in Cos and Ind samples indicating an occurring perturbation of intracellular calcium and ions mobilization, while THIO was significantly overexpressed in all

samples, best in Cos sample, indicating an occurring oxidative stress in all the wastewater samples.

Tumor promotion associated biomarkers: EF2, DPP3 and 14-3-3T, were selectively overexpressed in wastewater samples (**Fig. 4. 18.**). 14-3-3T, which has an anti-survival effect through negatively controlling the cell cycle and activating apoptotic cascades, was significantly overexpressed in Cos sample, explaining in part the observed cytotoxicity caused by the sample (**Fig. 2. 2.**); EF2 was enhanced in both Inlet and Outlet samples indicating an increase in protein synthesis.

The expression patterns of HSP70 and HSP90 (**Fig. 4. 19.**) were slightly modulated, but remain not statistically significant. The sensitivity of identified biomarkers towards real wastewater samples was much higher than that of the reference stress genes HSP70 and HSP90.

The differential expression of the identified biomarkers in different wastewater samples gave important information regarding the toxicological effects differentiating between each wastewater origin. The transcriptional activation of the identified biomarkers was observed in different wastewater compositions which suggest the possibility that some compounds in these wastewaters mixtures share similar mode of actions with LAS, increasing thereby the possibility of using these biomarkers for cumulative risk assessment.

5. Conclusions

In this study, we explained the outlines of LAS cytotoxic effect on human intestinal Caco-2 cells. In fact, LAS induced a time and dose dependent cytotoxicity in Caco-2 cells accompanied by an induction of oxidative stress followed by an excessive increase of intracellular calcium level. Our findings suggest the involvement of Thioredoxin, Calreticulin and HSP7C in the cell response to

this cytotoxic effect, so that they can serve as informative biomarkers of effect, reflecting LAS mode of action. At non cytotoxic Concentrations, LAS may have a tumor promotion effect on colon cancer cells through increasing cell proliferation. This effect was associated with the up-regulation of EF2 and DPP3 and the down-regulation of 14-3-3T, so they can serve as biomarkers of effect reflecting LAS mode of action.

These biomarkers could help in improving understanding of the toxicological effects and serving in more realistic and informative future risk assessment studies.

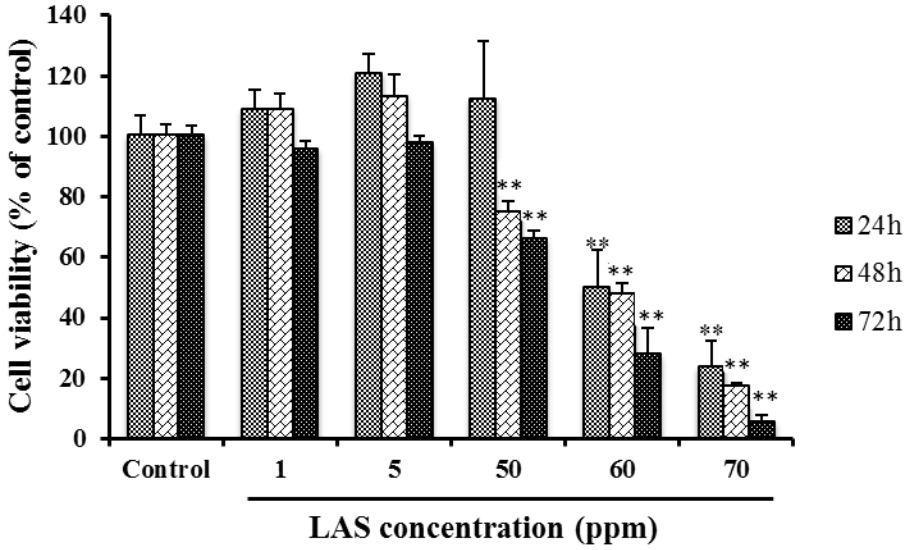


Fig. 4. 1. Reduction of MTT by Caco-2 cells after exposure to different LAS concentrations for 24 h, 48 h and 72 h; Cell viability was determined as the mean of absorbance at 570 nm and expressed as the percentage of the control; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

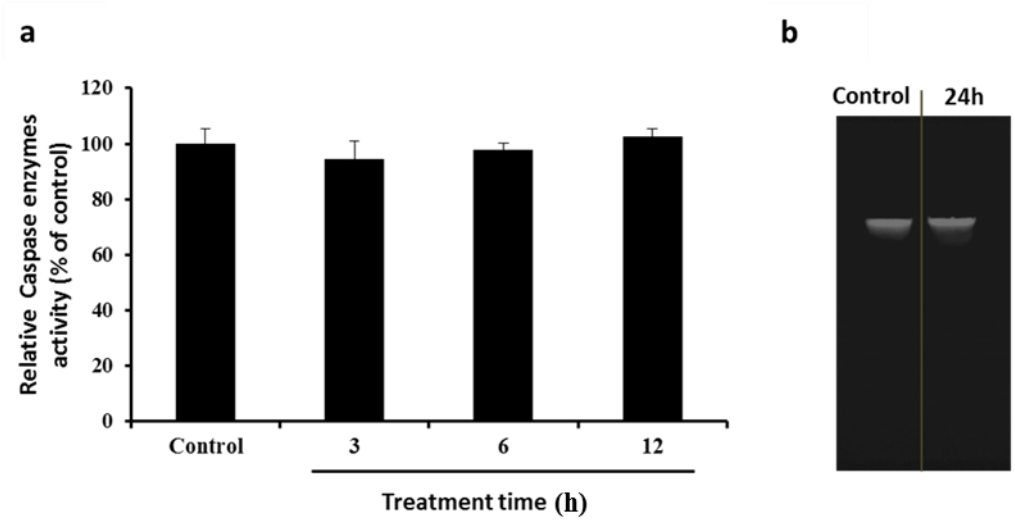
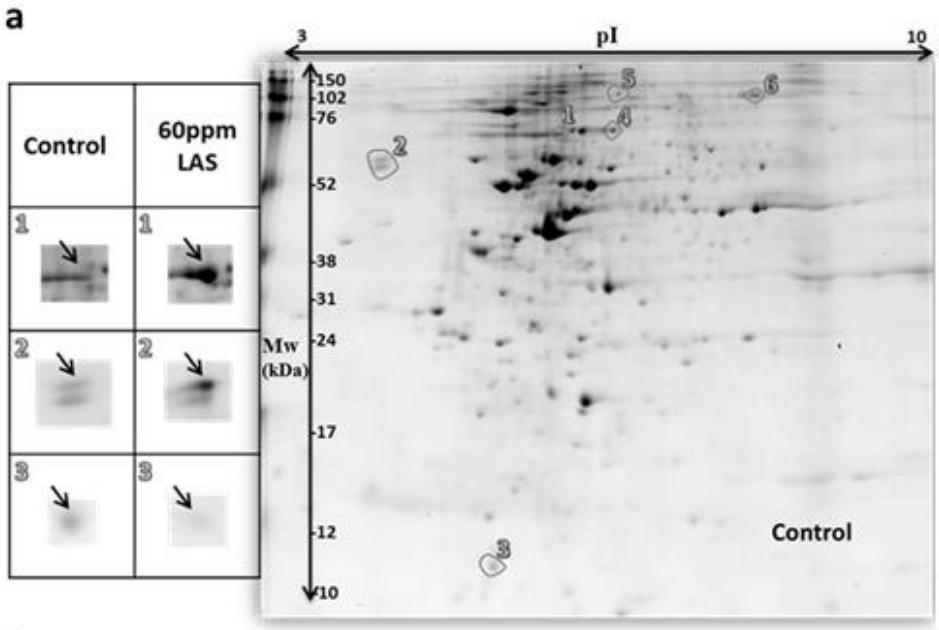
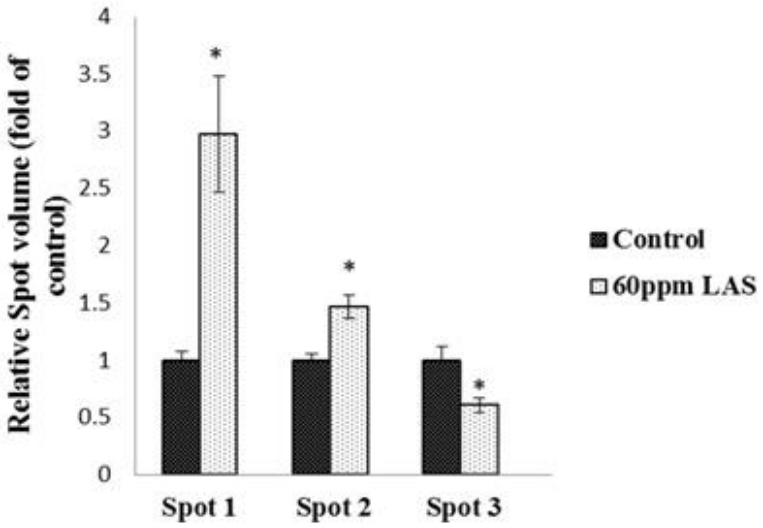


Fig. 4. 2. Effect of LAS exposure on caspase enzymes activity (a) and DNA aspect (b) in Caco-2 cells; Caco-2 cells were exposed to 60 ppm LAS for different times: 3 h, 6 h and 12 h for Caspase assay (a), and 24 h for DNA fragmentation assay (b); Control represent the non-exposed Caco-2 cells; Caspase enzymes activity is expressed as percentage of control; Extracted total DNA aspect was observed under ultraviolet illumination after staining with ethidium bromide. Data represent the mean±SD of three independent experiments


a
Control
60ppm LAS
1
2
3
pI
3
10
150
102
76
52
38
31
24
17
12
10
Mw (kDa)
Control


b
Relative Spot volume (fold of control)
4
3.5
3
2.5
2
1.5
1
0.5
0
Spot 1
Spot 2
Spot 3
Control
60ppm LAS

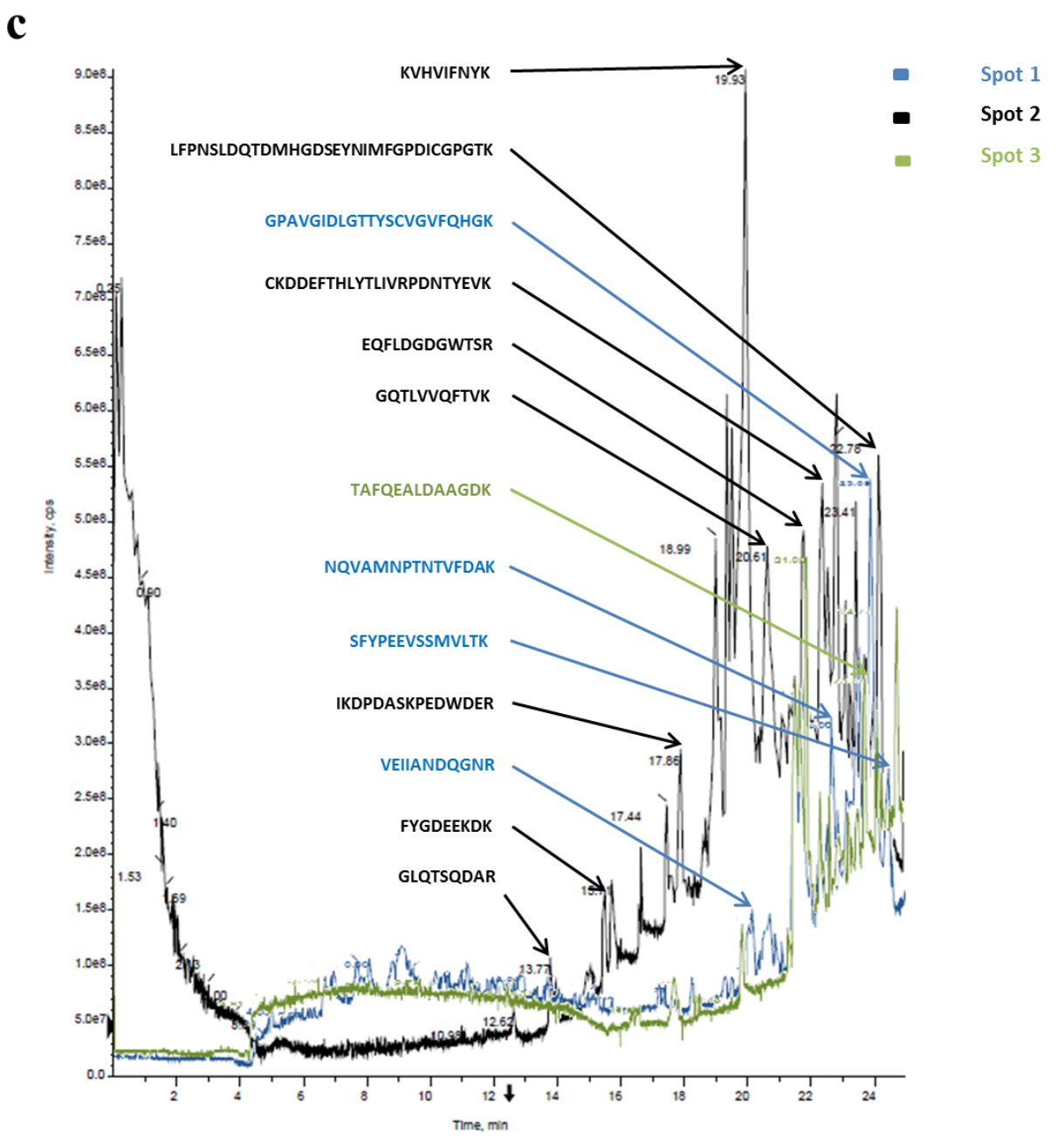


Fig. 4. 3. Representation of 2-Dimensional CBB-stained gel illustrating exemplary protein expression in Caco-2 cells with enlarged images of differentially expressed proteins after exposure for 24 h to 60 ppm LAS (a); Relative volumes (fold change of control) of the 3 differentially expressed protein spots (b); Spots volumes were obtained using ImageMasterTM

2D platinum software; Results represent the mean±SD of two independent experiments. (c) Tryptic digests spectra of the 3 selected biomarkers with the main identified peptide fragments;

* indicates significant difference from the control ($p<0.05$)

Table 4. 1. Summary of cytotoxicity-related proteins differentially expressed in Caco-2 cells exposed to 60 ppm LAS for 24 h

Spot no.	Accession no.	Score	Calculated *pI* / Observed *pI*	Calculated Mw / Observed Mw (kDa)	Protein name	Function category
1	P11142	318	5.37/5.50	71/65	Heat shock cognate 71 kDa protein	Chaperone/ Stress response
2	P27797	239	4.29/4.00	48/55	Calreticulin	Chaperone/ Ca^{2+} homeostasis
3	P10599	76	4.82/5.00	12/11	Thioredoxin	redox reactions

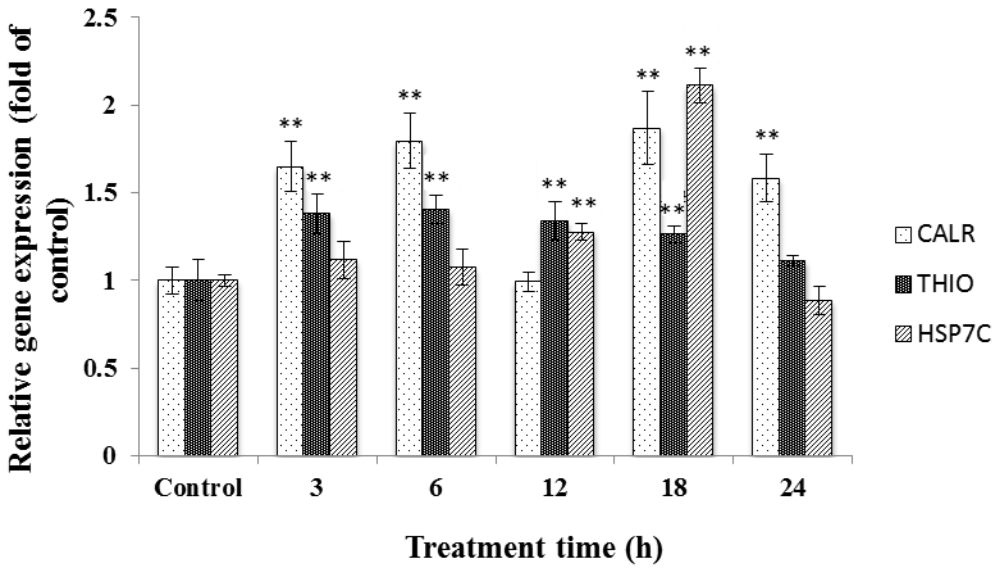


Fig. 4. 4. Relative gene expression of THIO (Thioredoxine), CALR (Calreticulin) and HSP7C (Heat shock cognate 71 kDa protein) in Caco-2 cells exposed to 60 ppm LAS for 3 h, 6 h, 12 h and 24 h. Data are expressed as fold-change of control (gene expression in non-exposed Caco-2 cells); *THIO*, *CALR* and *HSP7C* mRNA levels are normalized to *GAPDH* mRNA levels; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

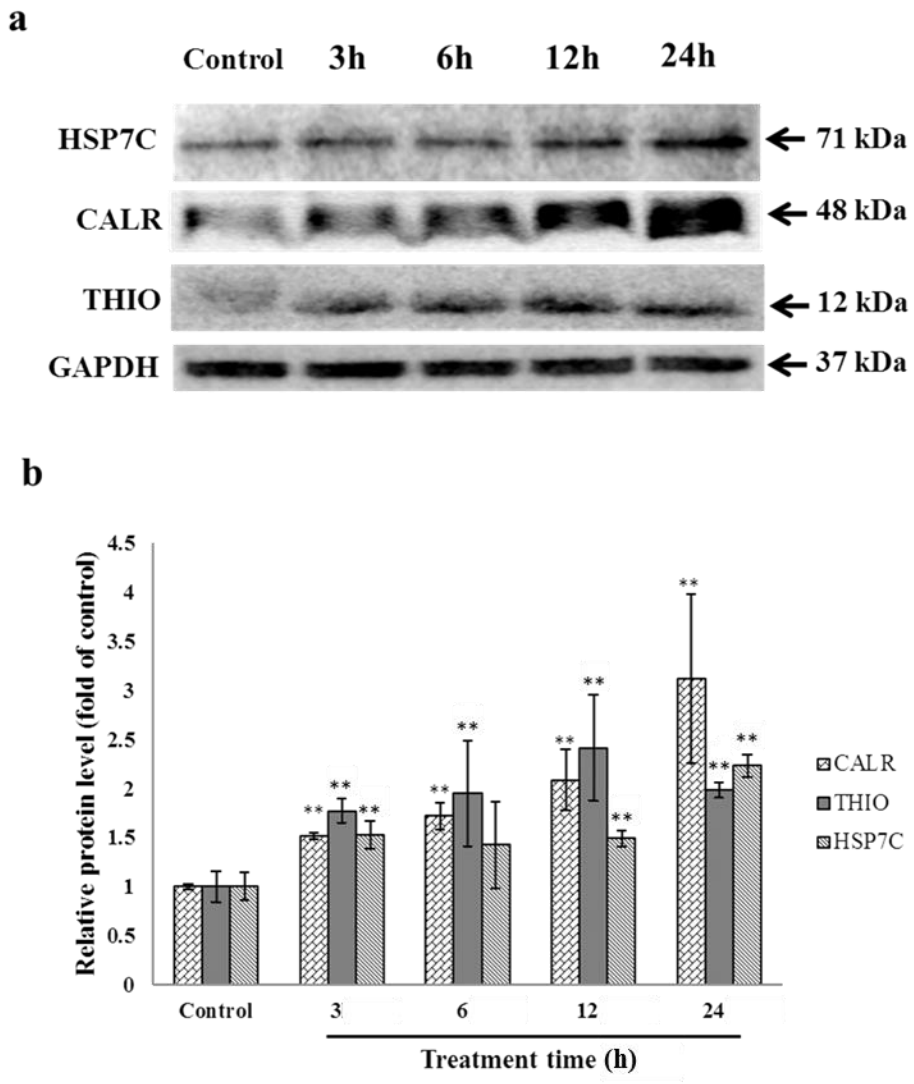


Fig. 4. 5. Relative protein expression of THIO (Thioredoxine), CALR (Calreticulin) and HSP7C (Heat shock cognate 71 kDa protein) in Caco-2 cells exposed to 60 ppm LAS for 3 h, 6 h, 12 h and 24 h. Control represent protein expression in non-exposed Caco-2 cells; GAPDH was used as loading control for Western Blot analysis; (a) Western blot images of THIO, CALR and HSP7C and GAPDH; (b) Relative protein level (relative bands intensity represented as fold of control); Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

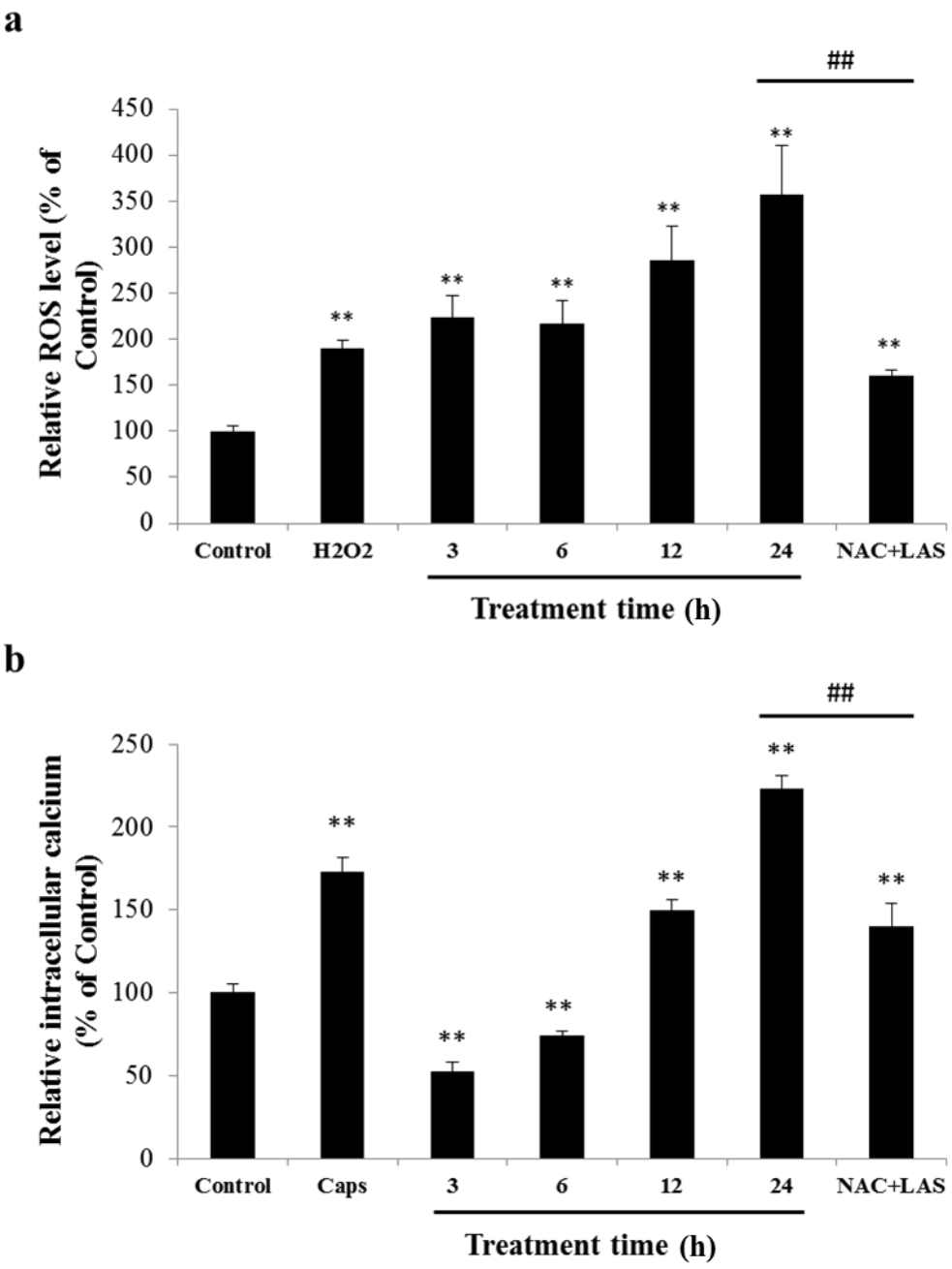


Fig. 4. 6. Relative ROS level in Caco-2 cells exposed to 60 ppm LAS for 3 h, 6 h, 12 h and 24 h, 100 µM H_2O_2 for 1h, and 1 mM N-Acetyl-L-Cystein+ 60 ppm LAS for 24 h (NAC+LAS) (a); Relative Intracellular Calcium level in Caco-2 cells exposed to 60 ppm LAS for 3 h, 6 h, 12 h and 24 h, 200 µM capsaicin for 1h, and 1 mM N-Acetyl-L-Cystein+ 60 ppm LAS for 24 h (NAC+LAS) (b); Results are expressed as % of control, where control represent non-exposed

Caco-2 cells; Data represent the mean±SD of at least three independent experiments; * indicates significant differences from the control, and # indicates significant differences from 24h treatment: * and # for $p<0.05$, ** and ## for $p<0.01$

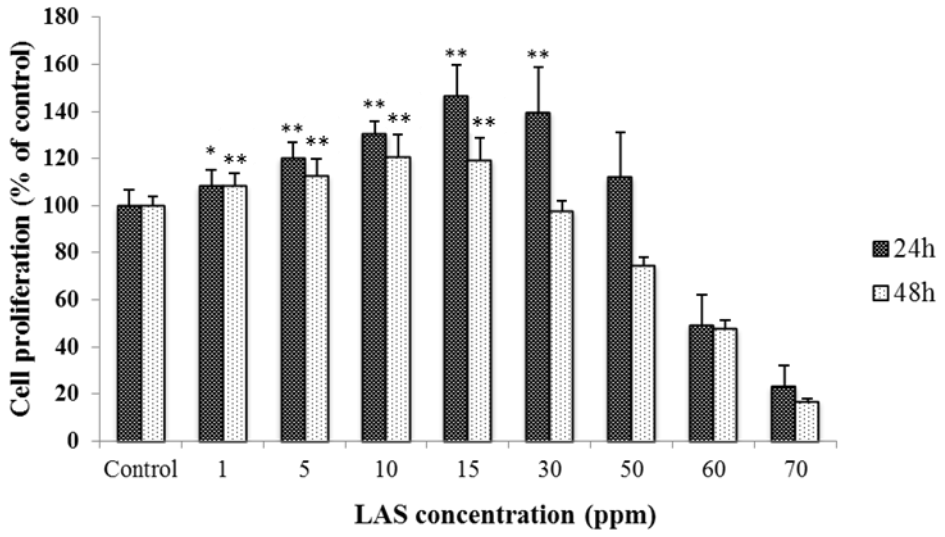


Fig. 4. 7. Reduction of MTT by Caco-2 cells after exposure to different LAS concentrations for 24 h and 48 h; Cell proliferation was determined as the mean of absorbance at 570 nm and expressed as the percentage of control; Results represent the mean±SD of at least three independent experiments; * indicates significant increases compared to the control: * for $p<0.05$; ** for $p<0.01$

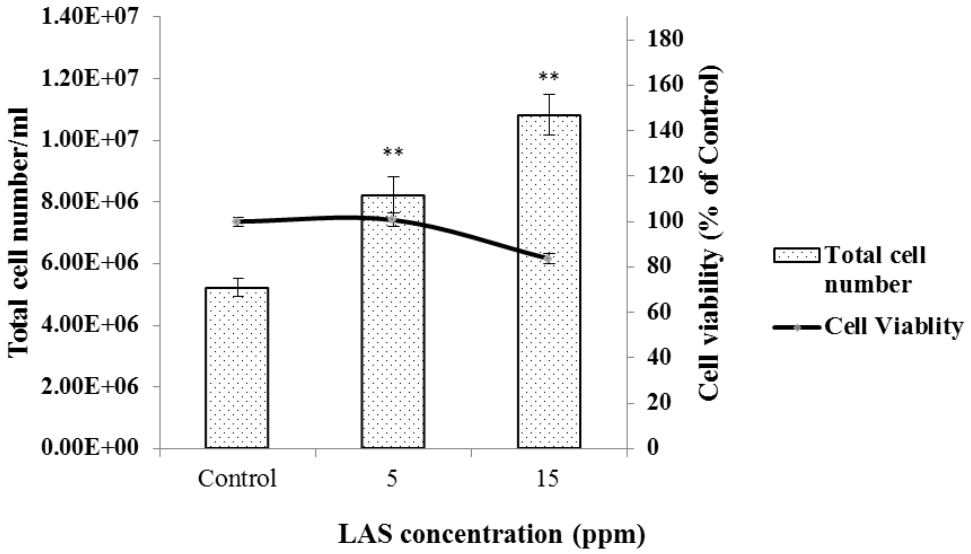


Fig. 4. 8. Cell number and viability of Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h; Control represents non-treated Caco-2 cells; Cell number and cell viability were measured by flow cytometry using Guava ViaCount reagent; Results represent the mean±SD of three independent experiments; ** indicates significant difference from the control ($p<0.01$)

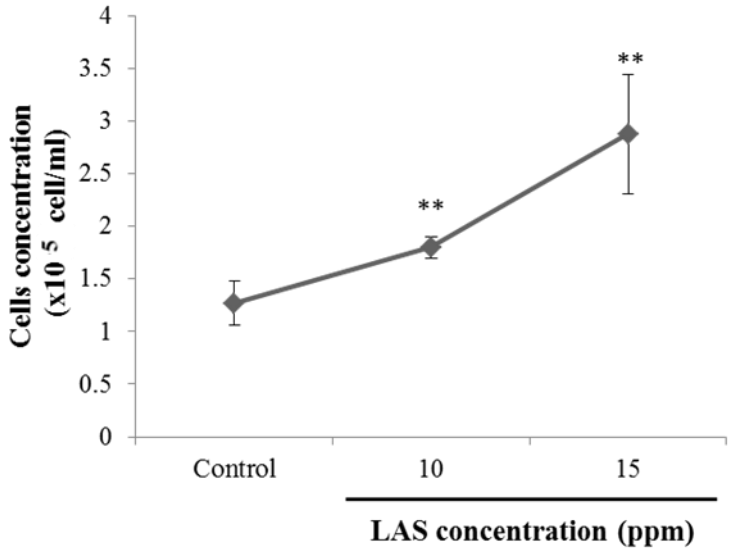


Fig. 4. 9. Cell number of non-exposed and exposed Caco-2 cells to 10 and 15 ppm LAS for 24 h; Control represents non-treated Caco-2 cells; Cell number was measured using trypan blue exclusion method; Results represent the mean±SD of three independent experiments; * indicates significant difference from the control: * for $p<0.05$ and ** for $p<0.01$

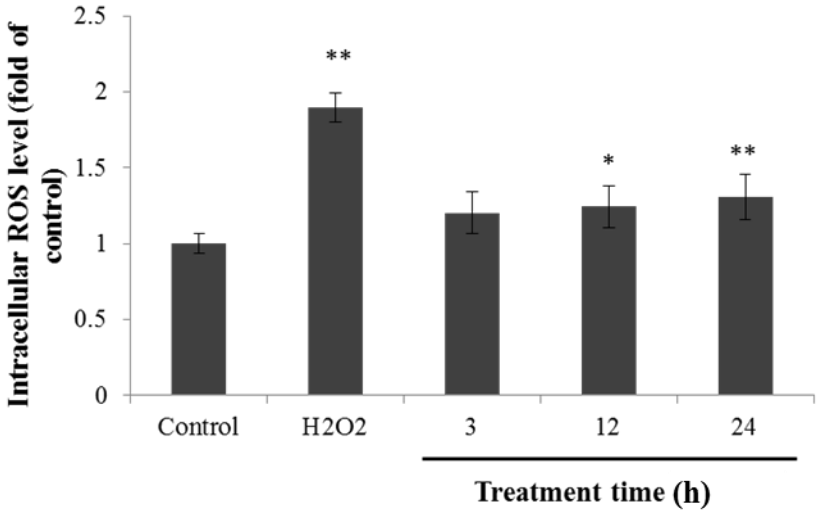


Fig. 4. 10. Intracellular ROS level in Caco-2 cells exposed to 5 ppm LAS for 3 h, 12 h and 24 h, and 100 µM H_2O_2 for 1h; Results are expressed as fold of control, where control represent non-exposed Caco-2 cells; Data represent the mean±SD of at least three independent experiments; * indicates significant difference from the control: * for $p<0.05$ and ** for $p<0.01$

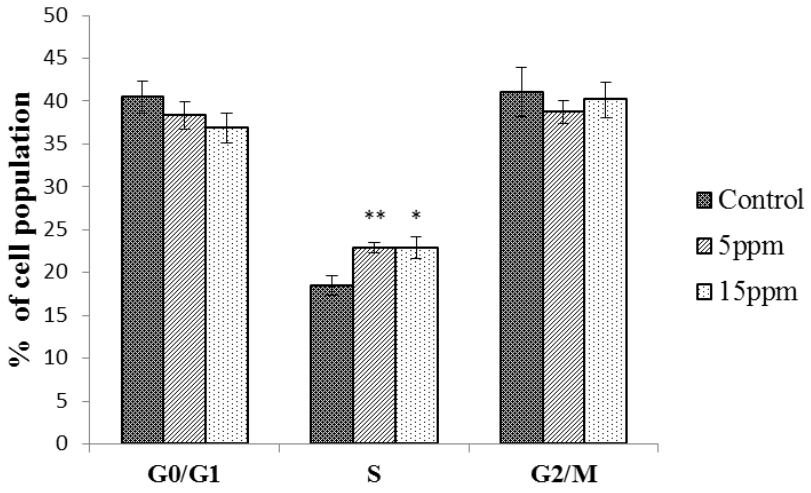


Fig. 4. 11. Cell cycle distribution of Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h; cell populations in G0/G1 phase, S phase and G2/M phase were determined by flow cytometry in both treated and non-treated Caco-2 cells and expressed as % of total cells; Results represent the mean±SD of three independent experiments; * indicates significant difference from the control: * for $p<0.05$ and ** for $p<0.01$

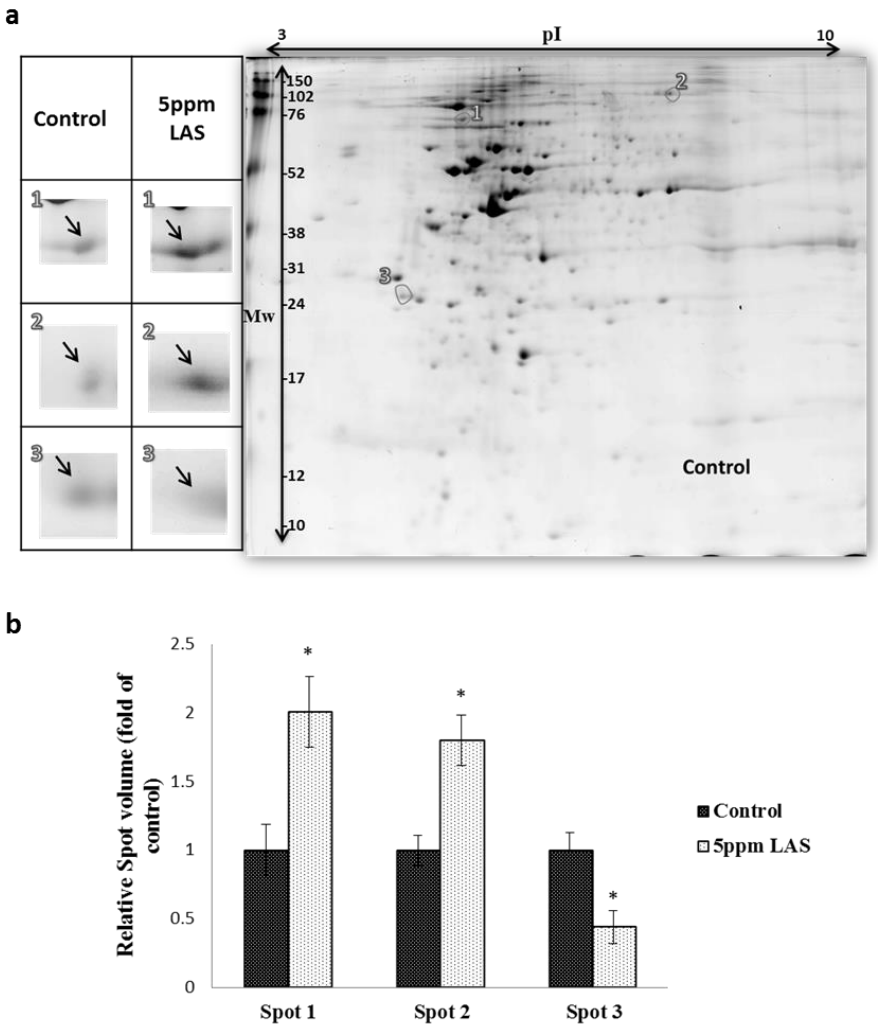


Fig. 4. 12. Representation of 2-Dimensional CBB-stained gel illustrating exemplary protein expression in Caco-2 cells with enlarged images of selected differentially expressed proteins after exposure to 5 ppm LAS for 24 h (a); Relative volumes (fold change of control) of the 3 protein spots (b); Spots volumes were obtained using ImageMasterTM 2D platinum software; Results represent the mean±SD of two independent experiments; * indicates significant difference from the control ($p<0.05$)

Table 4. 2. Summary of cell cycle and cell proliferation related proteins differentially expressed in Caco-2 cells exposed to 5 ppm LAS for 24 h

Spot no.	Accession no.	Score	Calculated *pI* / Observed *pI*	Calculated Mw / Observed Mw (kDa)	Protein name	Function category
1	Q9NY33	171	5.02/5.20	82/76	Dipeptidyl peptidase 3	Dipeptidyl-peptidase activity
2	P13639	404	6.41/7.00	96/102	Elongation factor 2	Translation elongation factor activity
3	P27348	155	4.68/4.75	28/26	14-3-3 protein theta	Regulation of signaling pathways

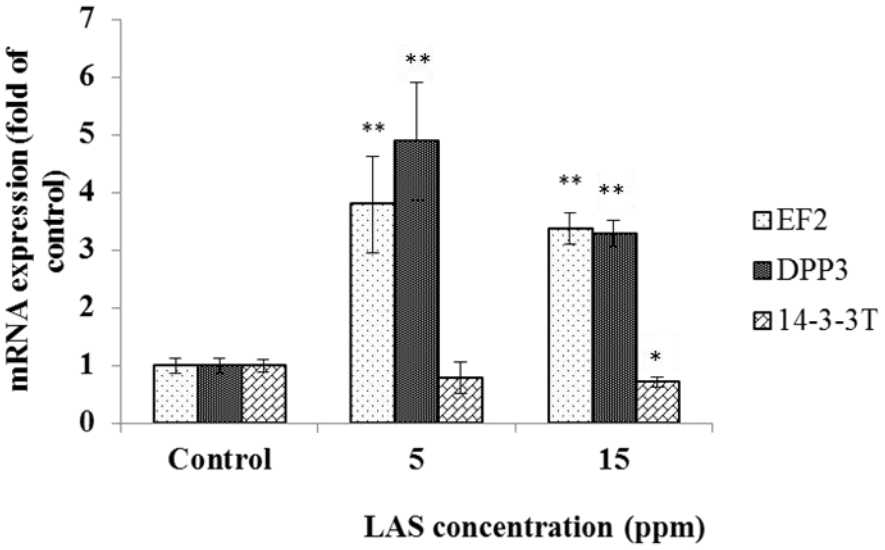


Fig. 4. 13. Relative gene expression of Elongation factor 2 (EF2), Dipeptidyl peptidase 3 (DPP3) and 14-3-3 protein theta (14-3-3T) in Caco-2 cells exposed to 5 and 15 ppm LAS for 24 h. Data are expressed as fold-change of control; *EF2*, *DPP3* and *14-3-3T* mRNA levels are normalized to *GAPDH* mRNA levels; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

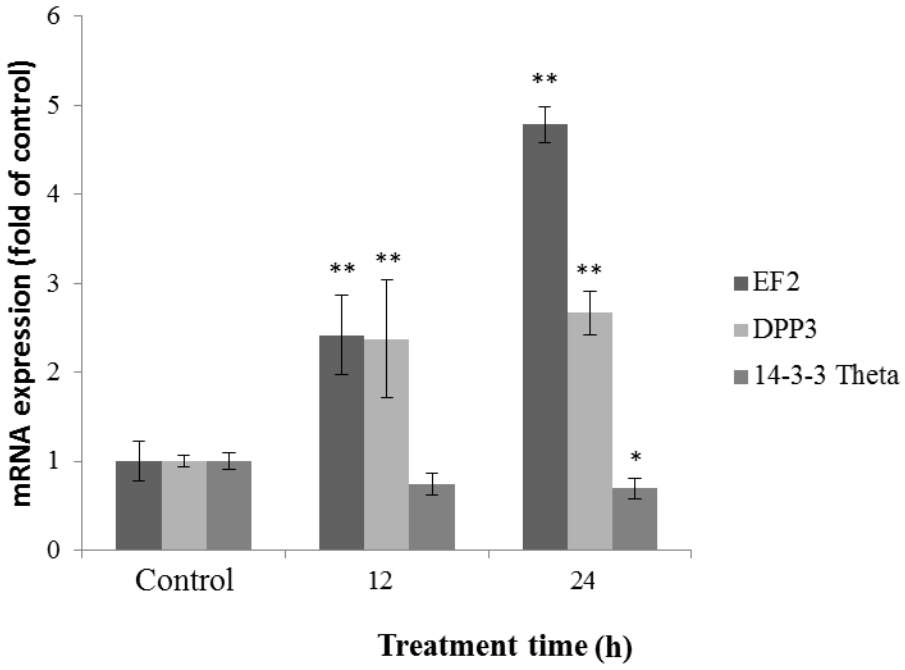


Fig. 4. 14. Relative gene expression of Elongation factor 2 (EF2), Dipeptidyl peptidase 3 (DPP3) and 14-3-3 protein theta (14-3-3T) in Caco-2 cells exposed to 60 ppm LAS for 12 and 24 h. Data are expressed as fold-change of control; *EF2*, *DPP3* and *14-3-3Theta* mRNA levels are normalized to *GAPDH* mRNA levels; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

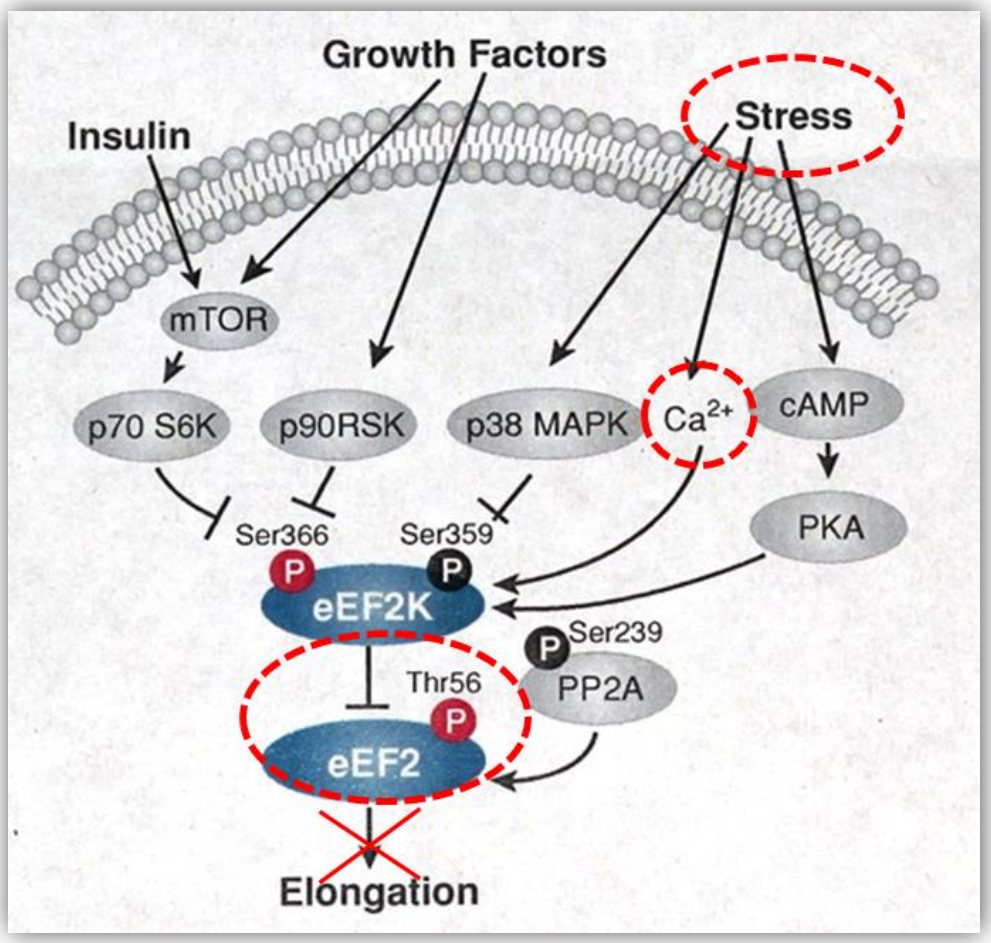


Fig. 4. 15. Phosphorylation and inactivation pathway of Elongation Factor 2

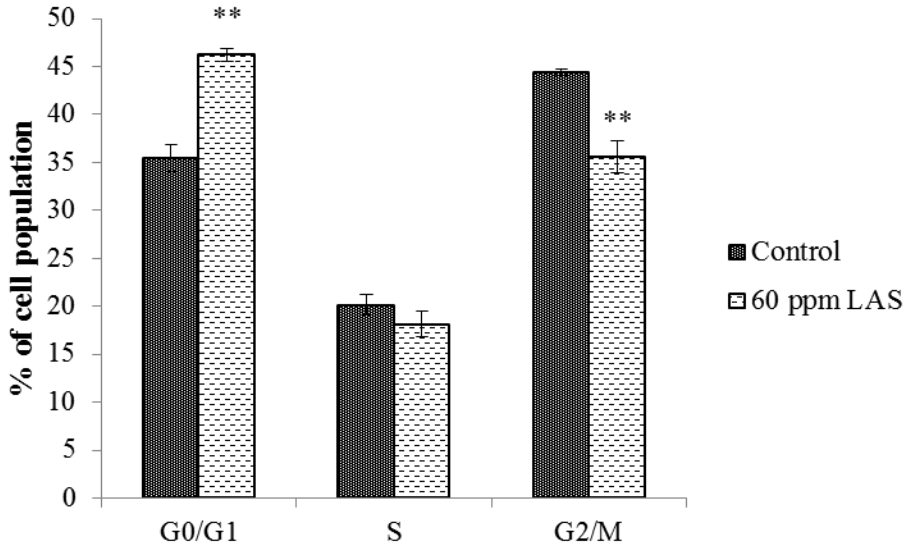


Fig. 4. 16. Cell cycle distribution of Caco-2 cells exposed to 60 ppm LAS for 24 h; cell populations in G0/G1 phase, S phase and G2/M phase were determined by flow cytometry in both treated and non-treated Caco-2 cells and expressed as % of total cells; Results represent the mean±SD of three independent experiments; * indicates significant difference from the control: ** for $p<0.01$

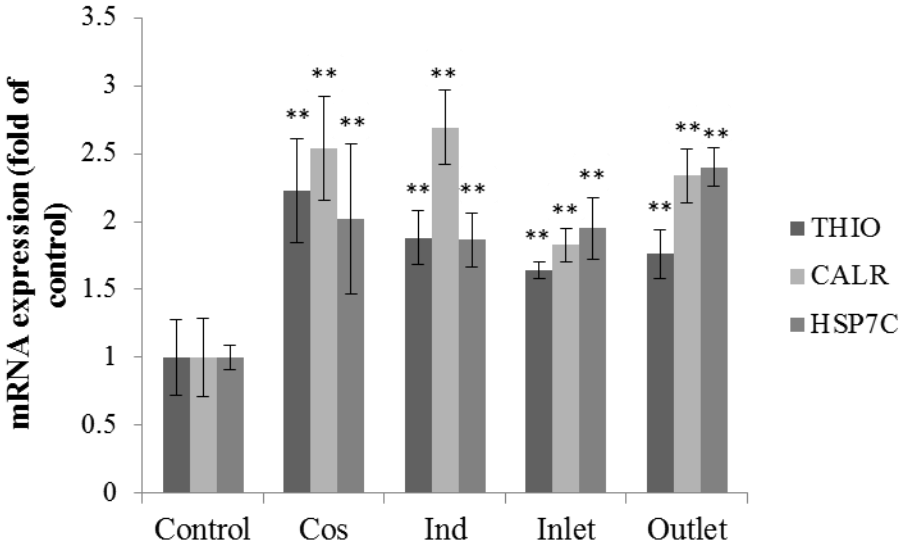


Fig. 4. 17. Relative gene expression of Thioredoxin (THIO), Calreticulin (CALR) and Heat shock cognate 71 protein (HSP7C) in Caco-2 cells exposed to 10% of different wastewater samples: Cos (Cosmetic wastewater), Ind (mixture of industrial wastewaters), and the inlet and outlet of southern Sfax municipal wastewater treatment plant (Inlet and Outlet), for 24 h. Data are expressed as fold-change of control; *THIO*, *CALR* and *HSP7C* mRNA levels are normalized to *GAPDH* mRNA levels; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: ** for $p<0.01$

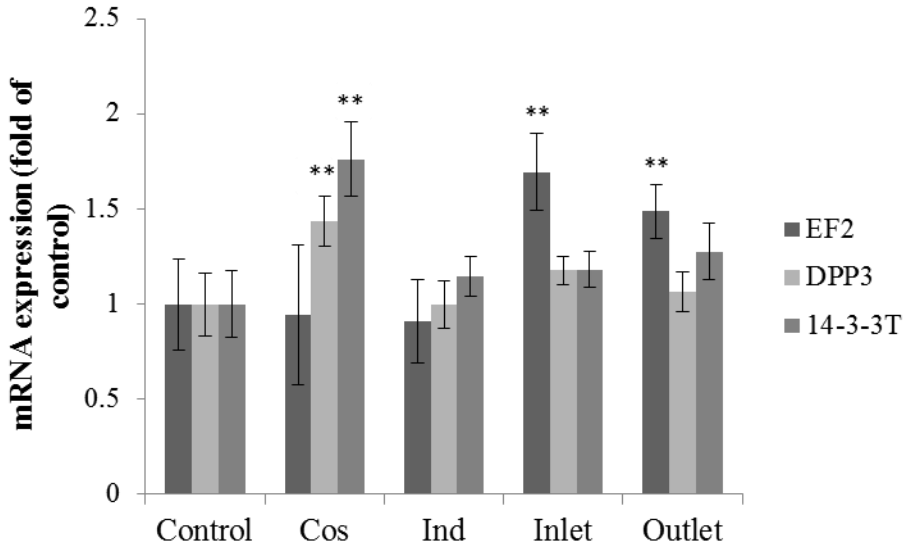


Fig. 4. 18. Relative gene expression of Elongation factor 2 (EF2), Dipeptidyl peptidase 3 (DPP3) and 14-3-3 protein theta (14-3-3T) in Caco-2 cells exposed to 10% of different wastewater samples: Cos (Cosmetic wastewater), Ind (mixture of industrial wastewaters), and the inlet and outlet of southern Sfax municipal wastewater treatment plant (Inlet and Outlet), for 24 h. Data are expressed as fold-change of control; *EF2*, *DPP3* and *14-3-3Theta* mRNA levels are normalized to *GAPDH* mRNA levels; Results represent the mean±SD of three independent experiments; * indicates significant differences from the control: * for $p<0.05$; ** for $p<0.01$

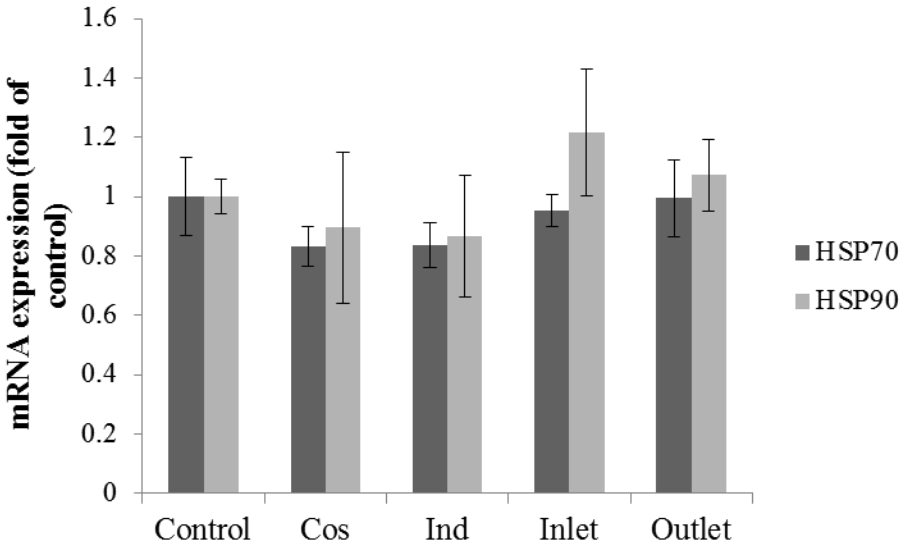


Fig. 4. 19. Relative gene expression of reference stress genes: HSP70 and HSP90 in Caco-2 cells exposed to 10% of different wastewater samples: Cos (Cosmetic wastewater), Ind (mixture of industrial wastewaters), and the inlet and outlet of southern Sfax municipal wastewater treatment plant (Inlet and Outlet), for 24 h. Data are expressed as fold-change of control; *HSP70* and *HSP90* mRNA levels are normalized to *GAPDH* mRNA levels; Results represent the mean±SD of three independent experiments

CHAPTER 5

General conclusions and perspectives

Anionic surfactants water contamination is an emerging form of water pollution, especially in the developing countries of arid and semiarid regions. To shed some light on this issue, in chapter 2, the author investigated the toxicological profile of different wastewaters from an industrial aria in Sfax, Tunisia, with special emphasis on the anionic surfactants levels. Moreover, the author attempted to ameliorate and optimize a wastewater treatment process for anionic surfactants removal, and tests on industrial scale gave good results that fit the standards specified by law.

Furthermore, the improvement of risk assessment process is crucial for more realistic and reliable assessment data. To overcome some of the limits of current procedure, recent calls recommend the use of toxicants mode of action as a potential solution to improve our understanding of toxicological effects and to allow the prediction of cumulative effects resulting from combined mixture effect (Clewell, 2005; National Academies Standing Committee on the Use of Emerging Science for Environmental Health Decisions, 2012).

In the present dissertation, the author attempted to construct a novel strategy for LAS (one of the major anionic surfactants used on the market) risk assessment, through establishing and incorporating molecular biomarkers reflecting the mode of action associated with its adverse effects.

In chapter 3, the investigation of LAS effect on Caco-2 cell line revealed two different toxicological outcomes depending on LAS concentration. In fact, at relatively high concentrations, LAS induced a cytotoxic effect in Caco-2 cells with an LC_{50} of 60 ppm for 24 h. While at relatively low non-cytotoxic concentrations, LAS increased cell proliferation of the Caco-2 colon cancer cells and showed a potential tumor promotion effect. Each of the two

effects was further studied in chapter 4, in order to identify appropriate informative biomarkers of effect for each (**Fig. 5.**).

In chapter 4, the author demonstrated that the cytotoxic effect of LAS is associated with the generation of oxidative stress via increasing ROS, and the disruption of the intracellular calcium homeostasis resulting in excessive raise in intracellular calcium levels. Accordingly, proteomics approach revealed three biomarkers associated with the toxicological effect in Caco-2 cells, which are Thioredoxin, Calreticulin and HSP7C marking respectively the oxidative stress, the intracellular calcium balance disruption and the cell death stress. Similarly, the author demonstrated that LAS-induced increased Caco-2 cell proliferation at non-cytotoxic concentrations was associated with a slight increase of ROS levels in exposed Caco-2 cells. Proteomics results revealed that the observed effect was associated with an over-expression of Elongation factor 2 and Dipeptidyl peptidase 3, and a down-regulation of 14-3-3 protein theta, implicated in cell proliferation, cell cycle and cancer. These findings suggest that LAS at non cytotoxic concentrations, similar to those observed at wastewater treatment plants outlets, has a potential tumor promotion effect on colon cancer cells through increasing cell proliferation and promoting cell cycle progression, involving Elongation factor 2, Dipeptidyl peptidase 3 and 14-3-3 protein theta. These proteins are proposed to be informative biomarkers reflecting the observed increased proliferation effect caused by LAS.

In order to get practical conclusions regarding the use of the identified biomarkers in risk assessment, the author attempted to incorporate them and investigate their sensitivity in the risk assessment of different wastewater samples obtained from an industrial zone in Sfax city, Tunisia.

The following general conclusions were consequently obtained:

- This study provided data explaining the toxicological effects of LAS on Caco-2 cells and gave insights into the mode of action of this compound for both effects: cytotoxic effect and tumor promotion effect at High and low non-cytotoxic concentrations respectively.
- The use of proteomics approach, in addition to different bioassays, revealed some informative Biomarkers associated with the mode of action of LAS, that will help in more reliable future risk assessment studies.
- The sensitivity of identified biomarkers was much higher than that of the reference stress genes HSP70 and HSP90, and also more informative (reflecting cellular effects). Thus, these biomarkers have great potential to be used in more reliable and informative future risk assessment studies.
- The differential expression of the identified biomarkers in different wastewater samples gave important information regarding the toxicological effects differentiating between each wastewater type.
- The transcriptional activation of the identified biomarkers was observed in different wastewater compositions, even for samples having negligible amount of LAS, which suggest the possibility that some compounds in these wastewaters mixtures share similar mode of actions with LAS, increasing consequently the potential of cumulative and synergistic effects, and suggesting the possibility of using these biomarkers for cumulative risk assessment.

Accordingly, the following perspectives are deduced form this work:

- The ability of biomarkers to be transcriptionally activated in response to different compounds can be of great importance for cumulative risk assessment, since they share, at one or many points, toxicological pathways.
- Toxicants sharing similar mode of actions are more susceptible to have cumulative or synergistic effects. Thus improving knowledge regarding toxicants mode of actions can help in the prediction of possible synergistic or cumulative effects.
- The mixture effects assessment still one of the most difficult challenges in risk assessment, however toxicants combinations are limitless, thus it is impossible to explore all mixtures without a clear strategy. In this respect, The establishment of a mode of action biomarkers database, classifying toxicants according to their mode of action, can refine the huge number of possible combinations, and help in the prediction of possible cumulative effects: “the more toxicants share same biomarkers, the higher chance they could have cumulative effects”.

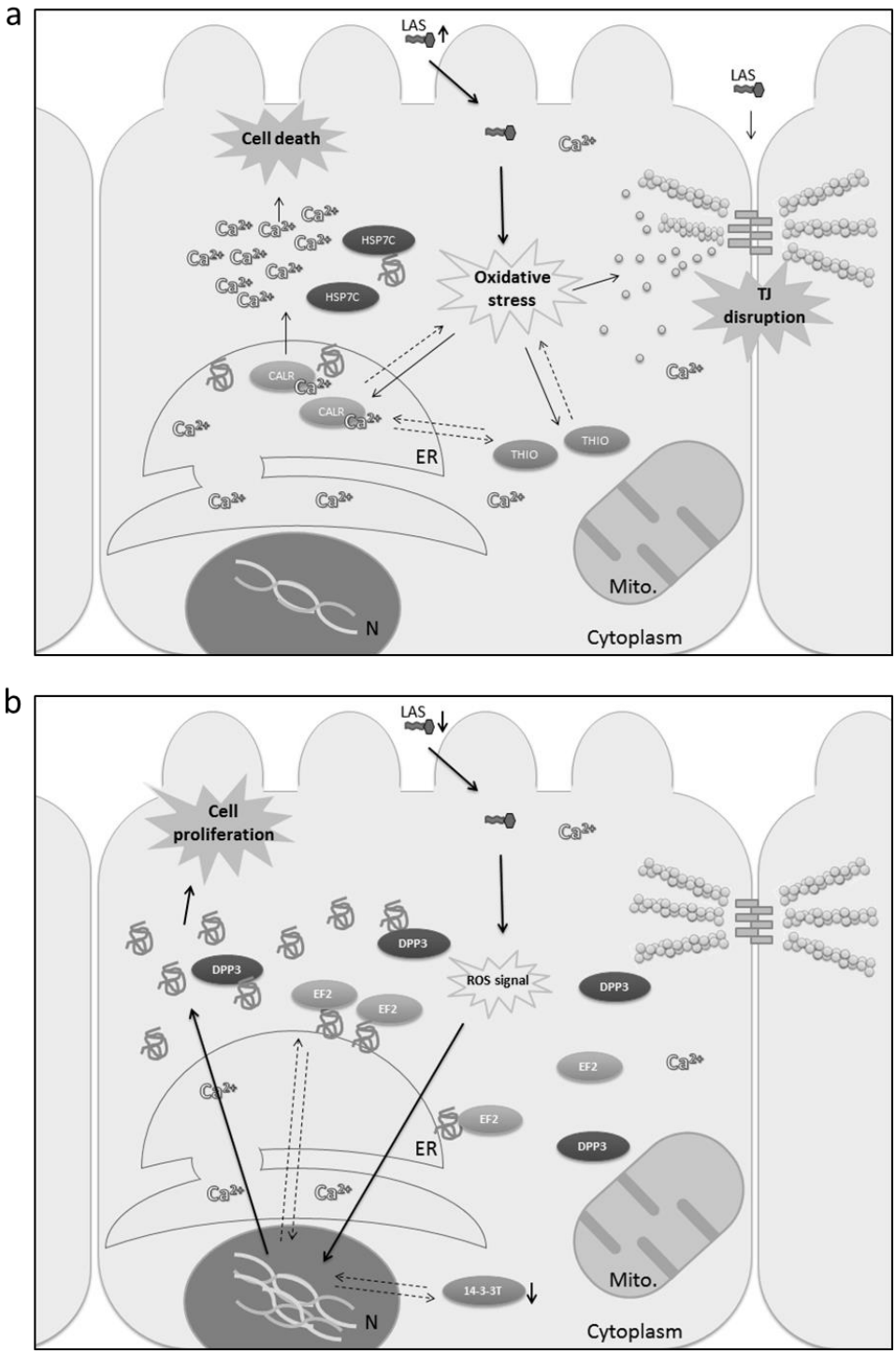


Fig. 5. Schematic summary of the adverse effects of LAS on Caco-2 cells showing the involvement of the identified biomarkers; (a) Cytotoxic effect at high LAS concentrations; (b) Proliferative effect at low non-cytotoxic LAS concentrations

SUMMARY

In, Arid and semi-arid regions, water resources are facing two major problems, a quantitative one, which is their scarcity, and a qualitative one aggravating the first, related to water pollution and contamination. In Tunisia, among the most common pollutants found in water media (especially wastewaters), surfactants take the lead from a quantitative point of view. Linear alkylbenzene sulfonates (LASs) are among the major anionic surfactants used in detergents such as, laundry powders and dishwashing products. After use, such detergent compounds are discharged into the environment in wastewaters, and contribute to the contamination of different environmental compartments (e.g. rivers, ground water, soils). Thus, the risk assessment of these compounds is of a great importance, especially that LASs are considered as toxic compounds to different terrestrial and aquatic living organisms.

In a first part, we tried to optimize a new method for the treatment of an industrial wastewater, highly loaded with anionic surfactants, especially LASs, emerging from a cosmetic industry in Sfax city in Tunisia. This method combines a physicochemical pretreatment by coagulation-flocculation using lime and alumina sulfate, with a biological treatment using MBR (Membrane BioReactor). The optimization was carried out using Response Surface Methodology (RSM) through a two factors central composite plan, to adopt the physicochemical pretreatment conditions for the smooth functioning of the whole process, in order to obtain treated wastewater that fit the Tunisian standard NT 106.02. The integration and optimization of a physicochemical pretreatment by coagulation-flocculation using lime and alumina sulfate, has allowed the good operating of the treatment process and the release of a treated surfactant-containing wastewater that fit the standards specified by Tunisian law.

Furthermore, the concentration of the pollutant alone does not reflect necessarily its real effect on the endpoint level, especially when considering interactions and cumulative effects in environmental pollutants mixture, suggesting that the focus should be on the effect rather than the concentration for more realistic risk assessment approach. However, the current procedure for environmental/human health risk assessment is interested in the concentration of the active substances taken separately. Accordingly, there is a need to develop new methods for assessing the actual risk of toxicants, which consider toxicological and cumulative effects resulting from exposure to a mixture of contaminants having similar mode of actions.

In the present dissertation, we attempted to construct a novel strategy for LAS risk assessment through establishing and incorporating molecular biomarkers reflecting the mode of action associated with its adverse effects. Following the new tendency of reducing the use of animals in toxicity tests and the encouragement for the use of human cell and tissue models, in addition to the very few studies examining the adverse effects of LASs on human cell lines, we investigated the adverse effects of LAS ($C_{12\ h25}C_{6\ h4}SO_3Na$) on human intestinal Caco-2 cells, which have been proven to be a valuable model for the study of cytotoxicity mechanisms. We tried, therefor, to understand the underlying mechanism using proteomics approach and different bioassays, in order to identify appropriate biomarkers, reflecting the mode of action, which can serve for more reliable and informative future risk assessment studies. The sensitivity of these biomarkers toward LAS-containing wastewater samples from industrial area in Tunisia was also studied.

As results, we demonstrated that LAS exerts two different effects on Caco-2 cells depending mainly on the concentration. In fact higher concentrations exert a time and dose dependent cytotoxic behavior, unlike the relatively low non-cytotoxic concentrations which have a proliferative effect. At high concentrations exceeding 50 ppm, LAS induces an oxidative stress

followed by an excessive increase of intracellular calcium level in Caco-2 cells, resulting in a cytotoxicity in time and dose dependent manner. Proteomics approach helped in discovering three informative biomarkers, first time to be reported, associated with LAS cytotoxic effect: Calreticulin, Thioredoxin and Heat shock cognate 71 (HSP7C), confirmed by real-time PCR and western blot analysis. Additionally, LAS increased Caco-2 cell proliferation at concentrations ranging from 1 to 15 ppm, more significantly for shorter exposure time (24 h). This effect was associated with an increase of cell population in S phase of the cell cycle, an over-expression of Elongation factor 2 and Dipeptidyl peptidase 3, and a down-regulation of 14-3-3 protein theta. All of the identified biomarkers were selectively transcriptionally activated after exposure to different LAS-containing wastewater samples from an industrial area in Sfax, Tunisia.

As conclusion, we explained in this study the outlines of LAS adverse effects on human intestinal Caco-2 cells. In fact, at relatively high concentrations (more than 50 ppm) LAS induced an oxidative stress followed by an excessive increase of intracellular calcium level in Caco-2 cells, resulting in a cytotoxic effect in time and dose dependent manner. Our findings suggest the involvement of Thioredoxin, Calreticulin and HSP7C in the cell response to this cytotoxic effect, so that they can serve as informative biomarkers reflecting LAS cytotoxicity mode of action. However, at low non-cytotoxic concentrations (1 ppm to 30 ppm), our data suggest that LAS may have a tumor promotion effect on colon cancer cells through increasing cell proliferation and promoting cell cycle progression, in association with the overexpression of EF2 and DPP3 and the down-regulation of 14-3-3T. These biomarkers could help in improving understanding of the toxicological effect and serving in more realistic and informative future risk assessment studies. Furthermore, the sensitivity of these identified biomarkers towards the wastewater samples was much higher than that of the reference stress genes HSP70 and HSP90,

nominating them to be good and more informative biomarkers for wastewaters risk assessment. Toxicants sharing similar mode of actions are more susceptible to have cumulative or synergistic effects. Thus improving knowledge regarding toxicants mode of actions can help in the prediction of possible synergistic or cumulative effects. Accordingly, the ability of the identified biomarkers to be transcriptionally activated in response to different compounds can be of great importance for cumulative risk assessment.

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